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A FURTHER STUDY OF EXPERIMENTAL PAROTITIS.

By MARTHA WOLLSTEIN, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 29.

(Received for publication, June 11, 1918.)

In a previous paper[1] it was shown that cats injected into the parotid gland and testicle with a bacterial sterile filtrate of the salivary secretion of children in the active stage of parotitis can be made to develop a pathological condition having several points of resemblance to that present in mumps in human beings.

The presence of acute cases of parotitis in military camps near New York City provided the opportunity to repeat the experiments with material from adult cases.[2]

EXPERIMENTAL.

Saliva from Patients.

In order to repeat the work done 2 years ago, mouth washings in normal saline solution were obtained from soldiers suffering from acute parotitis for 1 to 12 days. The washings were filtered through a Berkefeld candle N and the filtrate, which was sterile by ordinary aerobic and anaerobic culture methods, was inoculated into the parotid glands and testes of healthy, half grown cats.

There are several precautions to be observed in inoculating parotitis material into cats. The animals must be only half grown; old males do not react, even if they are well inoculated through the very tough

[1] Wollstein, M., *J. Exp. Med.*, 1916, xxiii, 353.

[2] I am greatly indebted to Captain Meader for placing material at Camp Mills at my disposal, and to Captain Monaghan and Lieutenants Cowdrey and Braiton for many courtesies at the Army Hospital at Secaucus, New Jersey. Material was also obtained at Camp Jackson, Columbia, South Carolina, through the kindness of Lieutenant Colonel Roberts.

connective tissue over the parotids on the side of the face. The most marked reactions, as expressed by severe symptoms and lesions, develop when the material is inoculated within a few hours after it is obtained from the patient; that is, as soon as it can be filtered. With proper care of the filters and the glassware, contaminations do not occur; and while the filtrates must always be tested for sterility, neither time nor animals are wasted by immediate inoculation if the technique is right.

Attempts to probe Steno's duct and obtain saliva directly from the swollen parotid were unsuccessful. The opening is normally small; during an attack of parotitis the mucous membrane is red and swollen, thus reducing the size of the orifice and making it even more difficult to introduce a cannula.

Filtrates of mouth washings obtained from patients ill 1 to 3 days produced characteristic symptoms and lesions in the inoculated cats, consisting of a rise in temperature, leukocytosis with an absolute increase in mononuclear cells, and tenderness and swelling of the injected glands with definite histological changes. All the symptoms appear after an incubation period of 5 to 8 days and endure about 8 days, when they decline.

PROTOCOL I.

Cat A.—Inoculated Nov. 23, 1917, with sterile filtrate made from the combined saliva of three soldiers who had been ill 36 hours. 2 cc. injected into the right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1917	°C.		
Nov. 23	38.9	23,000	Polynuclears 60; large mononuclears 18; small mononuclears 17; eosinophils 5.
" 24	40.0	25,000	No signs.
" 26	40.3	27,000	Polynuclears 70; large mononuclears 17; small mononuclears 11; eosinophils 2.
" 27	40.5	32,000	Cat is very quiet; ill.
" 30	40.8	33,000	Polynuclears 75; large mononuclears 16; small mononuclears 19; eosinophils 0.
Dec. 3	39.6	30,000	Less ill.
" 5	39.1	35,000	Polynuclears 63; large mononuclears 12; small mononuclears 19; eosinophils 6.
" 8	38.8	38,000	Polynuclears 71; large mononuclears 16; small mononuclears 9; eosinophils 4.
" 11	38.7	40,000	Polynuclears 71; large mononuclears 13; small mononuclears 11; eosinophils 5.
" 15	39.0	35,000	Chloroformed.

Right parotid fully twice as large as the left; the adjacent lymph nodes were swollen. The parotid was edematous, pink, and granular on section contrasting with the smaller, paler, and smoother left parotid. Cultures gave no growth. Microscopically the right parotid showed edema, some degree of mononuclear cellular infiltration around the ducts, and a less degree about the blood vessels.

Material was obtained from soldiers at Camp Jackson, Columbia, South Carolina, very early in the course of a parotitis attack. It was filtered in the usual way and inoculated into two cats. Both animals reacted well. Fig. 1 illustrates the parotid, submaxillary gland, and adjacent lymph nodes from one of the cats, removed after death on the 17th day.

On the 6th day of the disease the filtered saliva produced less marked symptoms, while inoculation of material obtained from a patient 9 days or longer after onset of the mumps attack was apparently without effect. The following protocol illustrates the latter point.

PROTOCOL II.

Cat B.—Inoculated Feb. 13, 1918, with sterile filtrate made from the saliva of a soldier at Secaucus, New Jersey; onset of mumps 10 days before. 2 cc. injected into right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Feb. 13	38.9	14,000	Polynuclears 56; large mononuclears 14; small mononuclears 20; eosinophils 0.
" 14	38.7	18,000	No symptoms.
" 16	38.6	12,000	Polynuclears 72; large mononuclears 12; small mononuclears 15; eosinophils 1.
" 18	38.8	13,000	Polynuclears 75; large mononuclears 6; small mononuclears 19; eosinophils 0.
" 20	38.7	17,000	No symptoms.
" 23	39.0	20,000	Polynuclears 73; large mononuclears 8; small mononuclears 19; eosinophils 0.
" 26	39.4	22,000	Polynuclears 75; large mononuclears 11; small mononuclears 14; eosinophils 0.
" 28	39.0	23,000	Chloroformed.

The right parotid was not larger than the left, and the adjacent lymph nodes were not swollen. Microscopic examination showed no lesion in either parotid.

It will be noted that in this animal the mononuclear leukocytes were actually decreased, and the polynuclear leukocytes slightly increased, a result which had been previously noted after the injection of saliva not containing the virus of parotitis.

Cats inoculated with material from soldiers on the 9th, 11th, and 12th days of an attack of parotitis gave results similar to those described in Protocol II; that is, the temperature range was only 0.5°C., the leukocytic count was not even doubled, the mononuclear cells were decreased instead of increased, the polynuclear cells were slightly increased, and at autopsy the parotid gland showed neither macroscopic nor microscopic changes. From this we may conclude that the period of infectivity of the mouth secretions, as far as this test is capable of indicating, is comparatively short, and covers about 1 week, corresponding to the swelling of the parotid. A fresh swelling appearing in the opposite parotid gland would, of course, prolong the infectious period for a given patient.

Saliva from Cats.

The saliva from inoculated cats was employed for further injection. By anesthetizing the animals large amounts of ropy saliva are readily obtained, and at the height of the symptoms, at the end of the 2nd week after inoculation, the saliva thus obtained was found to incite symptoms and lesions in other and normal cats. The salivary secretion from normal cats obtained in the same manner and injected gave only negative results.

Following the inoculation of infected materials not only was the parotid gland affected, but the submaxillary, the sublingual, and the adjacent lymph nodes also. The extent of the swelling of the salivary glands was greater in this series of experiments than in the first series reported.[1] Moreover, the lymph nodes on the uninoculated side were observed to be swollen and moist, as though the infectious agent present in the saliva had been carried to them, presumably by lymphatics, leading to reaction on the uninoculated side. On microscopic examination the swollen lymph nodes showed necrosis of the center of several lymph nodules, while the sinuses were distended and the lining cells swollen. In this connection it may be remarked that swelling of cervical lymph nodes was a marked condition in many of the soldiers ill with mumps.

As regards the lesions in the parotid glands and testes, it may be stated that they agreed with the descriptions given in the previous paper[1] and briefly are as follows: In the parotid there are edema of the interlobular connective tissue and mononuclear cell infiltration about the ducts, and occasionally about the blood vessels as well. Similar lesions have been described in the pathology of cases of human mumps. In the testis there is principally an epithelial degeneration resulting in diminished spermatogenesis.

Blood.

The marked constitutional symptoms observed in many patients with mumps suggested an investigation of the blood at the height of the attack.

Films were stained with Wright's and with Giemsa's stain. Cultures were made in the usual way and by the anaerobic (tissue ascitic

broth) method of Noguchi.[3] No parasites were detected under the microscope or cultures obtained. Blood was taken from a vein at the elbow in seven patients ill 2 to 3 days who were suffering from marked constitutional symptoms. The blood was defibrinated and had been proved sterile in glucose broth and on agar plates; it was inoculated into normal cats. The protocol of such an experiment is given, together with its control.

PROTOCOL III.

Cat C.—Inoculated Mar. 13, 1918, with defibrinated blood taken from the arm vein of a patient ill 3 days. 2 cc. injected each into right testis and right parotid.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 13	39.0	13,000	Polynuclears 67; large mononuclears 28; small mononuclears 5; eosinophils 0.
" 14	39.2	34,000	Polynuclears 80; large mononuclears 9; small mononuclears 11; eosinophils 0.
" 15	40.4	47,000	No signs.
" 18	39.2	35,000	Polynuclears 74; large mononuclears 20; small mononuclears 5; eosinophils 0. Right testis tender.
" 22	39.3	40,000	Testis tender.
" 26	39.5	43,000	Polynuclears 84; large mononuclears 4; small mononuclears 11; eosinophils 1.
" 28	38.8	40,000	Seems quiet and ill. Polynuclears 82; large mononuclears 6; small mononuclears 12.
" 30	39.0	38,000	Chloroformed.

Right parotid, submaxillary, and lymph nodes are swollen and pink and larger than the left (Fig. 2). Right testis also swollen. Microscopically the right parotid gland showed marked edema and swelling of the epithelial cells in some acini. The lymph nodes showed distention of the sinuses and swelling of the lining cells. The right testis showed a degree of characteristic epithelial degeneration.

[3] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

PROTOCOL IV.

Cat D.—Inoculated Mar. 19, 1918, with sterile defibrinated blood from a healthy adult man. 2 cc. injected each into the right parotid and right testis.

Date.	Temperature.	Leukocytes.	Differential counts and remarks.
1918	°C.		
Mar. 19	39.1	19,000	Polynuclears 60; large mononuclears 22; small mononuclears 18; eosinophils 0.
" 20	38.9	26,000	Polynuclears 72; large mononuclears 24; small mononuclears 4; eosinophils 0.
" 23	39.2	20,000	Animal well.
" 25	39.5	20,000	Polynuclears 72; large mononuclears 16; small mononuclears 12; eosinophils 0.
" 28	39.0	25,000	No signs.
Apr. 1	38.9	25,000	Polynuclears 65; large mononuclears 10; small mononuclears 25; eosinophils 0.
" 4	39.1	23,000	Chloroformed.

Both parotid glands of equal size; slight subcapsular erythrocytic discoloration in the right. No difference in the two testes.

An examination of Protocol III shows that while the temperature range was small it was somewhat greater than in Protocol IV; the blood count, on the other hand, showed a polynuclear leukocytosis in both animals, but while the mononuclear cells were diminished in the control cat they were actually increased throughout in the animal receiving the blood from the patient ill of parotitis. Identical results were obtained with the blood of four other patients which had been pooled before inoculation. The blood taken from a case with slight symptoms on the 5th day of illness gave no result on injection into a cat.

The conclusion drawn from this experiment is to the effect that the blood of parotitis patients, especially those suffering from severe constitutional symptoms, is infective for cats. This conclusion is in keeping with the clinical course and metastatic complications of certain cases. It may be remarked that one of the four patients whose blood was pooled suffered from orchitis. Meningitis has not infrequently been described, especially in the French literature, as complicating mumps. In one suspicious case among our series of patients, the spinal fluid was clear, contained no lymphocytes, and was without effect when inoculated into cats.

Recurrent Parotitis.

An opportunity was afforded of studying a case of so called recurrent mumps. The patient, a soldier, had a first attack in November, 1916. He came under my observation in March when he was suffering either from another attack or merely from an exacerbation, as the swelling had persisted somewhat since November. Cases of parotitis which recur after 3 or 4 weeks are not uncommon, and Jochmann[4] states that the swelling may endure for months or even for a year. In this patient the right parotid remained enlarged longer than the left; both had returned to about normal size in 6 months. Three sets of inoculations were made with the salivary secretion: one on March 13, one on May 1, and the last on May 15. The first two gave positive, the last a negative result. As regards the last it may be stated that the swelling of the parotid had disappeared soon after May 1. Fig. 3 shows the condition found in the glands after the second inoculation.

SUMMARY.

A new series of inoculations into cats of the filtered sterile salivary secretions derived from cases of parotitis has been described. They confirm the observations made in 1915–1916 and extend them to include the epidemic parotitis occurring in our military forces.

Incidentally confirmatory evidence of the filterable nature of the causative agent of mumps has been obtained.

It has been determined that the saliva of man and of inoculated cats, and the inoculated glands of the latter animals, contain the filterable, infective agent.

The lesions present in the inoculated organs conform to those described in our first publication. In addition, the lymph glands adjacent to the salivary glands on the uninoculated side were sometimes found to be swollen and to exhibit microscopic lesions. Probably the involvement resulted from salivary and lymphatic infection.

The "virus" of parotitis was detected most readily in the saliva during the first 3 days of the disease, less easily on the 6th day, and not

[4] Jochmann, G., Lehrbuch der Infektionskrankheiten für Aerzte und Studierende, Berlin, 1914.

at all on the 9th day. It was detected also in the blood of patients showing marked constitutional symptoms, and in the saliva of a case of recurrent mumps at the periods of enlargement of the parotid glands, but not 2 weeks after the swelling had subsided. It was not detected in the cerebrospinal fluid.

EXPLANATION OF PLATE 29.

FIG. 1. Parotid (*P*) and submaxillary (*SM*) glands with adjacent lymph nodes (*L*), showing swelling of the inoculated right side. Cat killed after 17 days.

FIG. 2. Parotid (*P*), submaxillary (*SM*), sublingual glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with blood from a patient ill 3 days. Cat killed after 18 days.

FIG. 3. Parotid (*P*), submaxillary (*SM*), sublingual (*SL*) glands, and adjacent lymph nodes (*L*), showing swelling of the right side inoculated with saliva filtrate from a recurrent case (second recurrence). Cat killed after 15 days.

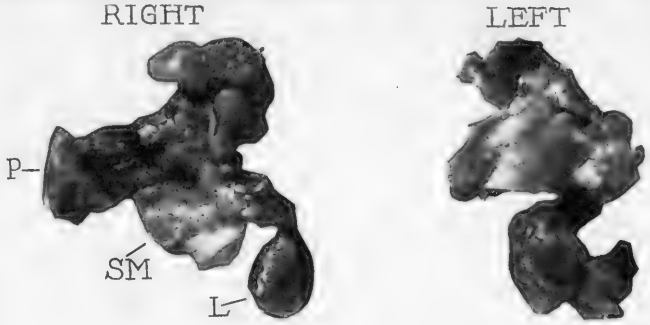


FIG. 1.

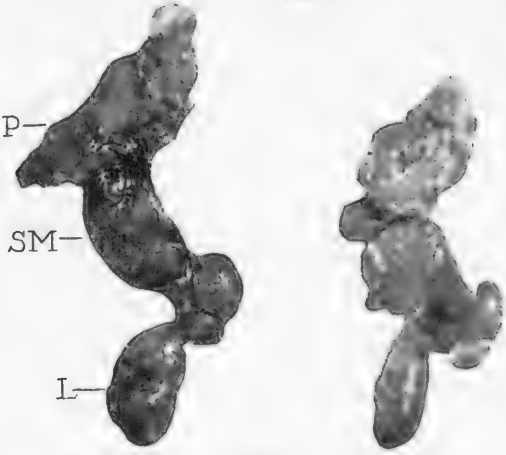


FIG. 2.

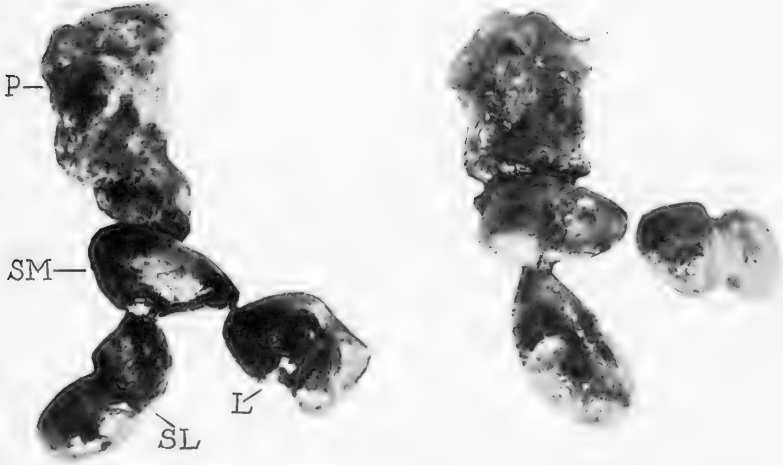


FIG. 3.

(Wollstein: Experimental parotitis.)

THE ENCYSTMENT OF DYSENTERY AMEBÆ IN VITRO.

By KAZUYOSHI YOSHIDA.

(*From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.*)

PLATES 30 TO 43.

(Received for publication, January 28, 1918.)

It has been stated in the literature that the vegetative form of the dysentery ameba dies within a few hours outside of its host and forms no cyst. In a study made in 1917, the author has observed, however, that the large vegetative ameba of dysentery is transformed into a smaller vegetative form, and that occasionally it develops *in vitro* a tetranuclear cyst. Proliferation takes place in fecal material containing blood and mucus. On the basis of these experiments we may conclude that the process of encystment of the dysentery ameba and change from the vegetative form proceed in nature in a manner similar to that observed in specimens taken from the host. The latter fact is most significant in the prophylaxis of amebic dysentery.

Material and Method of Investigation.

The previous finding that the dysentery amebæ obtained from bloody, mucous stools die if left to themselves is perhaps attributable to the fact that the bacteria also contained in the fecal matter proliferate and inhibit the life of the amebæ. We arrived at this theory on the basis of observations made in the cultivation of lung distomas. It was found that if tap water is added to the sputum containing the eggs of lung distomas, and the specimens are kept at a suitable temperature, with a daily renewal of upper water, in due time miradidiæ develop. If, on the other hand, the water is not renewed, it soon putrefies and the eggs die. In analogy with these observations on lung distomas, we have studied the organisms of amebic dysentery.

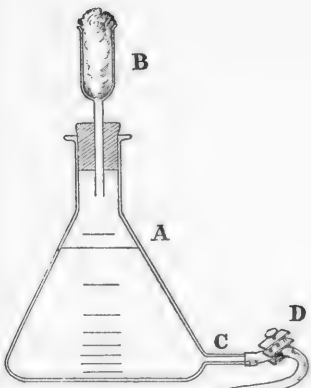
The material used for the investigation consisted of bloody, mucous stool obtained from a patient who had been under personal observa-

tion in the clinic for a number of years. Notwithstanding various methods of treatment, this patient never evacuated normally formed stools, his discharges being always soft, or bloody and mucous in character. It was possible to find in the feces numerous vegetative amebic forms, but a cyst was not observed, nor could we find other types of amebæ.

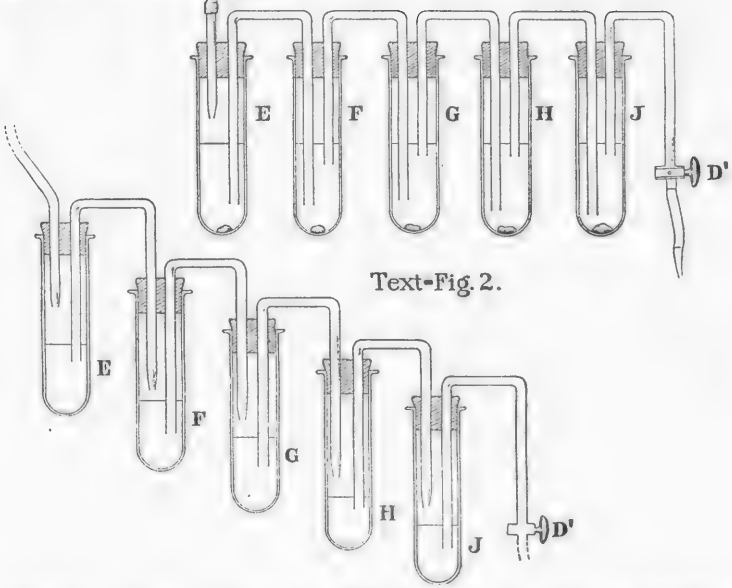
In the experiment various fluids were used as a medium; *i.e.*, tap water, Ringer's solution, isotonic salt solution, ascitic fluid, dilute bouillon, etc. These proved, however, to be unsatisfactory. One part of horse serum with four parts of Ringer's solution, occasionally with the addition of red blood corpuscles, gave good results.

The apparatus for the experiment was kept at a temperature of from 22° to 27°C. Under these conditions the multiplication of bacteria is relatively meager. The amebæ as a rule creep about in the medium. The ectoplasm is glassy in appearance, and there is less formation of vacuoles than at higher temperatures. The success of the study depends on the condition of the fecal matter. Bloody, mucous material containing numerous amebæ is most suitable. With the use of portions of formed stool, success is more difficult, possibly on account of the scarcity of amebæ and also because of the abundant intestinal flora present in such material. A small portion, the size of a hempseed, was deposited in a test-tube, and the fluid medium poured in by means of a regulated apparatus. When too large a particle of fecal material is taken the result is less satisfactory.

Text-figs. 1 and 2 illustrate the apparatus employed. *A* represents a reservoir, *B* a funnel with sterilized, burnt cotton plug, *C* a glass rod attached to a rubber tube (*I*), which carries the fluid from the reservoir. *D* is a stop-cock by means of which the velocity of the flow is controlled. *E*, *F*, *G*, *H*, *J* are smaller centrifuge tubes with rubber corks which, as shown in the drawing, are connected by means of glass capillaries. *D′* is a stop-cock to control the flow of the fluid when removing the tubes. The capillary which introduces the fluid is inserted into the lower depth of the tube, while that for drainage is inserted nearer the surface of the fluid. In this way it is possible to effect a renewal of the fluid, without draining off the amebæ. An apparatus constructed according to Text-fig. 1 is more satisfactory in this respect than that shown in Text-fig. 2.



Text-Fig. 1.



Text-Fig. 2.

TEXT-FIGS. 1 and 2. Apparatus employed for the cultivation of amebæ in fluid medium.

After sterilization of the entire apparatus, *A* was filled with the fluid medium. A small amount of bloody, mucous stool was deposited in each of the test-tubes and covered with the medium or Ringer's solution. All the tubes were then closed by rubber corks. The container was placed on a somewhat higher level than the test-tubes. When making the preparations, the desired test-tube was removed from the apparatus and the material taken from the bottom of the tube. Occasionally the material does not adhere well, so that it is necessary to place egg white glycerol on the cover-glasses.

The amebæ were studied in fresh preparations placed in a box which could be heated, and also in fixed and stained specimens. Sublimate alcohol was used for fixation, and iron hematoxylin for staining, according to the method of Heidenhain and Delafield. Examination of the amebæ in fresh and fixed preparations was made at intervals of 5 hours and later twice a day; *i.e.*, in the morning and the evening. It is necessary to have several sets of material in order to make consecutive daily examinations, for the supply is soon exhausted as the mass of fecal matter in the test-tubes is small.

Since January, 1917, twenty-five examinations have been made, nineteen of which were carried out according to the method described above. The viability of the dysentery amebæ fluctuated between 36 and 72 hours, the average being 51 hours, as shown in Table I.

Six experiments were carried out under anaerobic conditions, with the addition of 1 per cent boric acid as a medium, at body and room temperatures. The latter experiments, however, were unsuccessful.

Changes in the Morphological Properties of the Vegetative Form of Entamœba tetragena in a Fluid Medium.

The distinction between ectoplasm and endoplasm is clear in all stages. With unskilled technique, at a high temperature—which produces an abundance of bacteria—and with the use of various other fluid media, many of the amebæ show a markedly developed endoplasm, and a small and barely distinguishable ectoplasm. Sometimes the organisms have a large meshy structure, as in Fig. 2, owing to the presence of several vacuoles. This, however, is not the normal structure; it resembles that of the degenerative form of Hartmann.

TABLE I.

Duration of Life of Entamœba tetragena in Fluid Medium.

Experiment No.	Date.	Feces.	After 12 hrs.	After 24 hrs.	After 36 hrs.	After 41 hrs.	After 48 hrs.	After 55 hrs.	After 60 hrs.	After 72 hrs.	Remarks.
	1917										
1	Jan. 25, 2.30 p.m.	Bloody, mucous.	+	+	+	+	+	+	−	−	
2	" 27, 12.00 m.	" "	+	+	+	+	+	−	−	−	
3	Feb. 2, 3.00 p.m.	" "	+	+	+	+	+	−	−	−	
4	" 6, 11.00 a.m.	" "	+	+	−	−	−	−	−	−	Red blood cells added.
						Material poor.					
5	" 6, 5.30 p.m.	" "	+	+	+	+	+	−	−	−	
6	" 9, 12.00 m.	" "	+	+	+	+	+	+	+	+	
7	" 13, 10.40 a.m	" "	+	+	+	+	+	+	−	−	
8	" 16, 4 10 p.m.	" "	+	+	+	+	−	−	−	−	Died after 41 hrs. owing to temperature rise, 36°C.
9	" 17, 10.00 a.m	" "	+	+	+		−	−	−	−	
10	" 18, 10.40 "	Mucous.	+	−	−	−	−	−	−	−	Very few amebæ.
11	" 20, 4.30 p.m.	Bloody, mucous.	+	+	+	+	+	−	−	−	
12	" 22, 11.50 a.m.	" "	+	+	+	+	+	−	−	−	
13	" 24, 2.00 p.m.	" "	+	+	+	+	+	51st. +	−	−	
14	" 26, 5.00 "	" "	+	+	+	+	+	+	−	−	
15	Mar. 3, 10.00 a.m.	" "	+	+	+	+	+	51st. +	−	−	
16	" 4, 9.00 "	" "	+	+	+	+	+	+	−	−	
17	" 6, 11.00 "	Soft.	+	+	−	−	−	−	−	−	Very few amebæ.
18	" 16, 2.00 p.m.	Bloody, mucous.	+	+	+	+	+	+	−	−	
19	Apr. 2, 2.00 "	Soft.	−	−	−	−	−	−	−	−	No amebæ.

Movement takes place as a rule by means of hernial-sack-like pseudopodia. 5 hours after starting the experiment, particularly at body temperature, another mode of amebic movement is often seen. This consists in a forward motion in a definite direction by means of a single, homogenous pseudopodium.

Size of Amebæ.—Thirty amebæ in fresh and stained preparations were measured at different periods. It is well known that there are two vegetative forms of the dysentery ameba—the large and the small. The first is transformed into the smaller form and finally into the cyst. It is clear from the tables of the experiments made *in vitro* that all the amebæ decrease in size in the course of time, the reduction proceeding rapidly or slowly according to external and internal conditions, such as temperature, bacterial contents of the feces, viscosity of the medium, light, etc. Hence it is not possible to give average dimensions at certain periods of the experiment. The small amebæ are on the whole of uniform size; in fresh preparations they measure 13 to 15μ, the smallest being 7.5μ (Tables II and III).

The process of reduction in size may proceed in four ways as described below.

1. On the posterior side of an elongated ameba moving forward by a single pseudopodium the cell wall is observed to be missing, and the granular endoplasm appears to be in immediate contact with the outer medium (Fig. 14). Kuenen and Swellengrebel (1913) observed a similar phenomenon in the *histolytica* type of *Entamœba tetragena*. These authors, however, failed to interpret their observations. This posterior part of the ameba is viscous in character, a condition which serves well for the taking in of various nutrients, such as red blood cells and bacteria, and also for the expulsion of excreta. The process of ingestion can be observed readily in fresh preparations by placing with a fine pipette a drop of blood at the edge of the cover-glass. The reduction in size takes place by the extrusion of granules through this channel.

2. The mononuclear ameba becomes elongated and loses its motility, without, however, assuming a globular form; it is then constricted in the middle and divides in two. The part containing the nucleus resumes its movement and crawls along, while the other part finally disappears (Fig. 15, *a*, *b*, *c*, and Fig. 16).

TABLE II.

Morphological Changes in Entamœba tetragena in Fluid Medium.

Time.	In fresh preparations.				In stained preparations.						
	Size.	Distinction between ectoplasm and endoplasm.	Motility. Hernial-sack-like pseudopodium.	Motility. Flowing.	Size.	Size of nucleus.	Spherical shape.	Karyosome.	Reticulum of nucleus.	Chromatin outside nucleus.	Chromidia.
	μ				μ	μ					
Before experiment.	Maximum 33.0 Minimum 12.0 Average 26.5	+	+	+	Maximum 22.7 Minimum 13.4 Average 18.3	5.0 3.4 4.0	+	+	Mostly distinct.	+	−
After 5 hrs.	Maximum 27.0 Minimum 16.0 Average 20.5	+	+	+	Maximum 16.8 Minimum 11.8 Average 14.7	4.2 2.5 3.4	50%+	+	85% somewhat indistinct.	+	−
After 21 hrs.	Maximum 22.5 Minimum 15.0 Average 18.9	+	+	−	Maximum 13.4 Minimum 10.1 Average 12.0	5.9 1.7 3.7	20%+	Mostly +	Indistinct.	+	50%+
After 26 hrs.	Maximum 22.5 Minimum 11.5 Average 16.2	+	+	−	Maximum 15.1 Minimum 10.1 Average 11.8	5.0 2.1 3.7	83%+	+	Somewhat indistinct.	+	70%+
After 45 hrs.	Maximum 21.0 Minimum 7.5 Average 15.3	+	+	−	Maximum 13.4 Minimum 10.1 Average 11.2	4.2 2.5 3.5	75%+	Mostly +	Indistinct.	+	Mostly−
After 51 hrs.	Maximum 19.5 Minimum 9.0 Average 13.8	+	+	−	Maximum 14.3 Minimum 10.1 Average 12.5	5.0 3.4 4.2	65%+	+	Somewhat indistinct.	+	25%+
After 72 hrs.	Maximum 15.0 Minimum 7.5 Average 11.6	−	−	−	Maximum 12.6 Minimum 7.7 Average 10.5		Tetranuclear cyst formation.				

TABLE III.

Morphological Changes in Entamœba tetragena in Fluid Medium.

Time.	In fresh preparations.				In stained preparations.						
			Motility.								
	Size.	Distinction between ectoplasm and endoplasm.	Hernial-sack-like pseudopodium.	Flowing.	Size.	Size of nucleus.	Spherical shape.	Karyosome.	Reticulum of nucleus.	Chromatin outside nucleus.	Chromidia.
	μ				μ	μ					
Before experiment.	Maximum 34.5 Minimum 19.5 Average 27.4	+	+	+	Maximum 25.2 Minimum 20.2 Average 21.8	5.3 3.5 4.2	+	+	Distinct.	+	−
After 17 hrs.	Maximum 30.0 Minimum 10.5 Average 19.9	Mostly +	+	Occasionally +	Maximum 26.0 Minimum 12.6 Average 17.4	5.9 2.1 4.7	90%+	+	60% indistinct.	+	10%+
After 22 hrs.	Maximum 24.0 Minimum 15.0 Average 19.2	+	+	−	Maximum 21.0 Minimum 10.9 Average 15.3	4.2 3.4 3.9	85%+	Mostly +	85% indistinct.	+	15%+
After 41 hrs.	Maximum 22.5 Minimum 13.5 Average 18.1	+	+	−	Maximum 15.1 Minimum 11.8 Average 13.0	5.0 4.2 4.5	Mostly +	85%+	60% indistinct.	+	30%+
After 47 hrs.	Maximum 21.0 Minimum 15.0 Average 17 6	+	+	−	Maximum 12.4 Minimum 10.5 Average 11.8	5.0 3.4 4.1	60%+	+	Mostly indistinct.	+	60%+
After 51 hrs.	Maximum 21.0 Minimum 12.0 Average 16.0	+	+	−	Maximum 12.6 Minimum 8.4 Average 11.1		Nuclei pyknotically stained.				

3, *a*. A bud-like structure is occasionally seen at one or several places on the surface of an ameba in the process of nuclear change. These buds separate from the main body through the action of pseudopodia (Figs. 17 to 19).

3, *b*. A similar procedure takes place in the small forms, in particular shortly before death or encystment. When the conditions of life are unfavorable, the amebæ become globular in form and immobile. Masses of protoplasm at various points on the surface become constricted and separate from the main body (Figs. 20 to 22). The peripheral chromatin zone of the nucleus becomes gradually broader and the nucleus extrudes large masses of chromatin into the cytoplasm. These particles of chromatin in the cytoplasm increase until they finally fill the entire plasma in the form of chromidia. At the same time the degeneration of the nucleus takes place. The degenerated nucleus lies near the periphery, and has the appearance of a faintly stained flat disk or an irregularly shaped nuclear membrane. The constricted globules measure from 3 to 8μ in size, and are fairly refractile. They are at times yellowish in appearance, contain vacuoles, and are hyaline or granular in structure. The colored specimens on the whole take hematoxylin stain well and sometimes show chromatin masses (Figs. 21 and 22).

This process resembles the description given by Schaudinn and Hartmann on the cyst formation of *Entamœba histolytica*. Dr. Akashi of our clinic and other workers have also observed this reduction in the tetragenous ameba.

4. Before encystment the ameba divides into two daughter amebæ. It is possible that this is a mode of reproduction, but as the organism is reduced in size during the process, we have included it here.

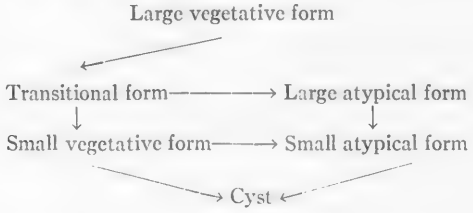
Six main forms can be distinguished with respect to the behavior of the cytoplasm and the nucleus.

Typical forms.
1. Large vegetative form.
2. Vegetative transitional form; *i.e.*, in the process of nuclear change.
3. Small vegetative form.

Atypical forms.
4. Large form.
5. Small form.
6. Cyst.

Forms 2, 3, 4, and 5 may be subdivided into chromidia-containing and non-chromidial organisms.

The following diagram shows the transitional stages of the large vegetative form into the cyst.



Large Vegetative Form.—As already stated, this is found in bloody, mucous stools. The nucleus usually shows the characteristic of the typical *Entamœba tetragena* as described by Hartmann. It appears as a spherical vesicle and has a distinct karyosome. On the inner side of the nuclear membrane are many small chromatin granules. The achromatic zone outside the nucleus has a meshy linear structure in which chromatin bodies are imbedded. The chromatic substance is found frequently at the outer edge of the karyosome area, in the middle of which a centriole is visible (Figs. 1 and 28 to 31).

Intermediate Form.—A few hours after starting the experiments a morphological change takes place in the organisms, and particularly in the nucleus. This has been called the intermediate stage or the stage of nuclear change (Figs. 2 to 4). The size of these organisms varies from 18 to 30μ.

In fresh preparations the nucleus appears enlarged, since in the course of the amebic movement it becomes somewhat flattened; no such enlargement, however, is observed in fixed and stained preparations. On the inner side of the nuclear membrane a definite increase of chromatin takes place, which makes the nucleus more conspicuous and the periphery more distinct (Fig. 52). The nucleus readily changes its form with the plasmic flow. There is an increase in the number of granules in the endoplasm, but no increase in the number of vacuoles. The motion is at first active, but decreases gradually. The amebæ are frequently found in compressed masses.

In stained preparations the nucleus is, as a rule, spherical, at times pear-shaped, infrequently polygonal. It is usually located

eccentrically on the border-line of ectoplasm and endoplasm. As a rule it is rich in chromatin. Sometimes the nucleus takes a diffuse intense stain, but the karyosome, nuclear membrane, etc., are distinct when the preparation is decolorized. The enlargement of the karyosome is characteristic of this stage. In the center appears a centriole. One may observe various stages of the cyclic process (Figs. 32 to 49). The chromatin outside the nucleus is particularly well developed. It occurs in particles and crescent- and ring-shaped bits. The meshy network of the nucleus is at this stage rather indistinct. The cytoplasm stains brown with hematoxylin and eosin, because of the chromatin which has migrated from the nucleus, in consequence of the cyclic process.[1]

Small Vegetative Form.—After from 15 to 25 hours, the forms described above are changed into small vegetative amebæ (Figs. 53 and 54). These organisms, found in the stools during convalescence from amebic dysentery, have been classified as belonging to the small vegetative form by Dr. Akashi of this clinic. Kuenen and Swellengrebel have referred to them as the "*minuta*" form. The organisms measure 10 to 18μ.

Large and Small Atypical Forms.—Occasionally we find among the intermediate and the small vegetative forms, organisms with atypical cytoplasmic and nuclear characteristics. The changes in the nucleus of these amebæ are particularly conspicuous. The large organism which develops from the intermediate form has been called the large atypical form (Figs. 55 to 59), and that evolving from the small vegetative form, the small atypical ameba (Figs. 60 to 62). The large atypical form then proceeds to change into a small atypical ameba. These two forms are identical with respect to the structure of the nucleus and cytoplasm, with the exception that the small atypical form is sometimes transformed into the cyst, while the large atypical ameba does not pass into this stage directly, but is first reduced to the small atypical form. Hence we may find the picture of cell division more frequently in the former than in the latter.

[1] The morphology of the amebæ described here coincides with Hartmann's illustrations (*Arch. Protistenk.*, 1910, xviii, 207, Fig. 11, *a*, *c*, *d*) of *Entamœba histolytica*. At this stage one sees numerous atypical nuclei.

The nucleus undergoes a characteristic change under conditions unfavorable for the life of the ameba. It shrinks or becomes enormously expanded, and assumes a polygonal, dumb-bell, crescent, or horseshoe shape. No particular shape of nucleus can be given as characteristic. In some cases the centriole is missing and the karyosome is hypertrophied (Figs. 55, 60, and 50). Sometimes one sees nuclei in which the karyosome gradually becomes indistinct or in which a trace of it only remains, together with a nuclear membrane relatively rich in chromatin (Figs. 57, 58, and 61). Again one sees amebæ with an hypertrophied polygonal karyosome in an environment rich in chromatin and the nuclear membrane hardly recognizable (Figs. 51 and 56). Soon one can no longer find the nucleus but only a mass of chromatin in the cytoplasm. The pseudopodia of such structures are often visible in stained preparations. Between this atypical and the typical cell structure lie various transitional pictures.

The two atypical forms show in fresh preparations the differentiation of the ectoplasm from the endoplasm. During motion broad lobed pseudopodia are formed which consist of hyaline ectoplasm. In fixed preparations the cytoplasm always shows a meshy structure, irregularly distributed. That of the large atypical form is coarser and less distinct than that of the typical amebæ. The cytoplasm of the small atypical form stains more deeply than that of the normal forms.

The morphology of the atypical amebæ here described corresponds completely with that given by Schaudinn, as well as with Hartmann's reproductions of *Entamœba histolytica*. Our amebæ resemble the degenerative form of *Entamœba tetragena* of Hartmann, but are distinguished from his by the kind of motility and the differentiation of ectoplasm from endoplasm. They never manifest motion in one direction as described by Hartmann for his degenerative form. Hence we have termed them atypical. In such forms chromidia formation is frequently present.

Chromidia and Cyst Formation.—The intermediate forms, the small vegetative forms, and the two atypical forms often become chromidial organisms in the course of extruding chromidia into the cytoplasm.

The intermediate and the large atypical forms are changed into large chromidial organisms (Figs. 73 to 76, 122, and 128); and the small vegetative and atypical forms, into small chromidial organisms (Figs.

7, 77, 78, and 124). The cell changes which have been described in the previous section are in the main to be regarded as a preliminary stage in the formation of chromidia, viewed from the standpoint of nuclear division. It is evident that the nucleus shows during this stage various conditions, *i.e.* slight changes (for example, in the nucleus of the intermediate form and the small vegetative form) up to degeneration (for example, in the nucleus of the atypical form), inasmuch as the chromidia arise through great accumulation of nuclear chromatin in the cytoplasm, and the formation of chromidia always takes place under conditions in the environment unfavorable to the ameba. The nucleus loses its spherical shape and assumes, as stated above, an indefinite form through being pulled about by the streaming of the cytoplasm. We have here probably atypical, rather than typical forms. The chromidial types of the vegetative forms do not, however, seem to show marked changes of the nucleus; 5 hours after staining no pronounced differences can be observed in the nuclear structure.

The chromatin is accumulated in a mass on the inner nuclear membrane, and the nucleus, as the figure shows, assumes various shapes. The chromatin mass migrates through the nuclear membrane in small particles into the cytoplasm (Figs. 3 and 76). In addition there are numerous other pictures in which the chromatin mass of the nucleus appears indistinct, the dividing line of the separate chromatin particles is not clear, and the cytoplasm stains intensely with hematoxylin. The structure is no longer finely reticulated, but fibrous or of a coarse mesh, and the cytoplasm contains variously stained rod- and spindle-shaped chromidia (Figs. 59, 74, and 75). It is clear that these are not the so called degenerative chromidia of Hartmann because of the paucity of vacuoles, the nature of the nucleus, and motion by means of pseudopodia. The chromidia may be divided into the following types: (1) those present in the cytoplasm in the shape of rods; the number varies between one and several dozen (Figs. 56 and 76); (2) small and irregularly formed chromidia, in a mass, comma-shaped, wedge-shaped, in zigzag formation, or globular, and located in the vicinity of the nucleus (Figs. 70 to 73, and 77); (3) two or three structures of considerable size, such as thick rods, wedges, and spindles (Figs. 7 to 9, and 78).

Although it is impossible to classify the chromidia definitely with respect to form and size, on the whole those of Type 1 seem to appear in the intermediate forms, *i.e.* in the first stage of chromidia formation; those of Type 2, in the two atypical forms; and those of Type 3 in the small vegetative forms having typical nuclei, and in the cyst forms. The point of origin and the significance of the chromidia are not yet clear.

Werner affirmed that the karyosome participates in the formation of the chromidia of *Entamœba histolytica*, and brought out many illustrations on this point. Hartmann agreed with this view, but claimed that the majority of Werner's illustrations showed degenerative and not genuine chromidia formations, and for that reason the amebæ having such chromidia could not form cysts.

The word "degenerative" so frequently employed by Hartmann is difficult to understand. This author in the first part of his work (1910) established from Schaudinn's preparations and preparations of dysenteric amebæ brought from China, the existence of *Entamœba histolytica*. In the second part he writes as follows:

After repeated study of Schaudinn's as well as my own preparations of *Entamœba histolytica*, I have come to the conclusion that with the exception of one case, we have here degenerative forms of *Entamœba tetragena*. This conclusion confirms an earlier surmise.

He did not, however, go into details concerning the exception of which he wrote. It seems clear that he could not deny the existence of *Entamœba histolytica*. The degenerated form, according to Hartmann, is an abnormal ameba possessing a degenerated nucleus and destined to destruction.

We may say, therefore, according to the view of Hartmann, that this abnormal form does not undergo transformation into the normal with a typical nucleus. Simultaneously with the disappearance of the nucleus, the substances given off by it may later form a new daughter nucleus of typical structure and the ameba remains in the vegetative form if environmental conditions are relatively favorable. But when conditions are unfavorable, the ameba dies without forming the typical nucleus; or a cyst having a typical nucleus is formed from the nuclear substances, and one to four daughter nuclei are developed. One cannot, therefore, say that the ameba which has a degenerated nucleus is a degenerative form. The majority of the "degenerative" specimens of Hartmann are not genuinely of this form. We believe them to be, on the other hand, atypical forms of *Entamœba tetragena*.

Hartmann also claims to have established that *Entamœba tetragena*, as well as *Entamœba coli*, always divides mitotically. He states:

Several nuclear divisions and schizogony, as in *Entamœba coli*, were never observed in *Entamœba tetragena*. On the other hand, we frequently observed nuclear pictures in which the chromatin was accumulated in a few large particles on the nuclear membrane. Such pictures, however, have nothing to do with multiple nuclear division, but are merely the expression of cyclic processes, or as we shall see later, an indication of degeneration.

Concerning nuclear division in the degenerative forms, Hartmann says:

These forms are, as a rule, smaller than the normal, which is obviously attributable to the rapid division of the amebæ at this stage and to the fact that the largest number of stages of division was found in such cases.

But Hartmann has not described the manner in which division proceeds in the nucleus without a karyosome. It is inconceivable that the nucleus divides by mitosis, inasmuch as no karyosome is present. We believe that this is not a case of mitosis, but a kind of amitotic division, or a primitive mitosis as described below.

As far as the degenerative chromidia are concerned, these can be interpreted in a similar manner. In our opinion the nuclei of dead cysts are often changed into chromidia. It is, of course, certain that the genuineness of these chromidia is not incontestable. Hence we believe that it is almost impossible to judge whether or not the chromidia of living amebæ are genuine, inasmuch as the chromidial formation always occurs under environmental conditions unfavorable to the ameba. The behavior of the cytoplasm likewise cannot be taken as a criterion in determining degeneration. Hartmann probably believed our atypical forms to be degenerative, for the reason that he considered only a mitotic, and not an amitotic or primitively mitotic division. It is also impossible to say whether or not the chromidia of Werner are degenerative in character, as affirmed by Hartmann.

Cyst Formation of Entamœba histolytica.—The writer is in doubt concerning the occurrence of the so called cyst formation of *Entamœba histolytica*, as emphasized by Hartmann, but regards this phenomenon rather as an atypical process of *Entamœba tetragena*. As already indicated in the reduction phase of *tetragena*, one sees an entirely similar procedure in the intermediate and the two atypical forms. Hartmann claims to have observed in this process increase in the amebæ of *Entamœba histolytica*, but no enlargement in *Entamœba tetragena*. His findings on *histolytica* coincide with ours in regard to the large

chromidial organisms. According to our observation, the amebæ did not increase in size, but the formation of chromidia took place in the case of relatively large amebæ. Hartmann's misinterpretation is probably due to the fact that he examined only the feces of patients, and was not able to follow as closely as we did the various developmental stages.

Fusion of More than Two Amebæ.—In accordance with the findings of Hartmann and Werner regarding *Entamœba histolytica*, we have observed also in the large chromidial organisms the fusing of two amebæ. This process is illustrated in Figs. 23 to 27. One of the organisms may show a rather markedly changed nucleus, while the other retains a well preserved nucleus. The two protoplasms of different specimens vary in their staining reactions. Whether this is a phenomenon of conjugation or plasmogony is not clear. While the author has never observed a fusion of more than three individuals, he has seen frequently in the small, vegetative, and small, chromidial organisms a thin protoplasmic stem holding together more than three individuals and also the protrusion of a pseudopodium from each organism. The cytoplasm of all the individuals varied in respect to staining, formation of chromidia, and vacuoles. The nucleus in all these instances showed sometimes a typical and again an atypical structure (Figs. 79, 124, and 129). It is highly probable that this is not a case of plasmogony, but rather of rapid cell division. This view is based on the picture of budding (Fig. 116).

From the above facts, however, we may conclude that in the majority of the forms of *Entamœba histolytica* described by Hartmann, we have really transitional and atypical forms of *Entamœba tetragena.*

Genesis and Significance of the Central Vacuole and Chromidia.—We are of the opinion that the central vacuole is frequently present during the encystment of *Entamœba tetragena*, as in other amebæ, while Hartmann claims that this is not the case. The vacuole is frequent in mononuclear cyst formation, particularly at the time of marked chromidia formation (Figs. 12 and 13), and disappears in mature cysts. Its genesis is not yet clear. In the small vegetative forms there is a peculiar formation of the chromidia and central vacuole.

On the 3rd day of the experiment, *i.e.*, toward the end of the vegetative stage, the chromidial organisms are seen (Figs. 67 and 127).

The nucleus is as large as that seen in the small vegetative form, and its structure is always atypical. At times it is difficult to distinguish the nuclear membrane, on account of the accumulation of chromatin. About the nucleus appears a broad area, which judging by its structure resembles expanded network of the zone outside the nucleus. The area frequently shows at the periphery a membranous structure, which differentiates the fine meshed cytoplasm from this zone. On the proximal side are found a number of large crescent-shaped chromatin masses. In this area with its coarse, spongy network one finds large rod-like or wedge-like chromidia. Our first impression was that this structure as a whole constituted an enormously enlarged nucleus, with the real nucleus a karyosome, and having in its center a centriole. Subsequently, however, upon finding various intermediary forms, it became clear that this light zone is not part of a nucleus, but probably a central vacuole.

It is interesting to find several intermediate stages between these structures and the ordinary chromidial organisms. The illustrations of Figs. 64 to 75 have been arranged to show in succession different stages of completeness. At first the usual chromidial organisms predominate. After a time, the other structures described above make their appearance. Fig. 66 shows the separation of the cytoplasm as a result of its flow from the zone. One can readily conceive that a membrane is formed at the surface of the area. The author has observed this phenomenon twice in stained preparations and once in a fresh preparation. It may possibly be interpreted as a form of endogenous cell formation of the ameba; *i.e.*, a new organism possessing a second nucleus arises from the original individual as in the schizogony of *Entamœba minuta* described by Popoff. Fig. 68 shows that an organism of this kind has independent motion by means of a pseudopodium protruding from the cytoplasm. One may observe a degenerated nucleus and a small newly formed nucleus in the clear zone. Fig. 63 shows an ameba which has two such nuclei of equal size, one of which is atypical in structure and the other of the newly formed type. Fig. 118 represents a cyst within which is an enormously enlarged nucleus (central vacuole) and four daughter nuclei which originated from the outer chromatin. Fig. 64 is a cyst produced under abnormal conditions. The central vacuole containing the nucleus has been freed by a

tearing away of the plasma. Its coarse network remains intact, and the central vacuole is changed into a cyst. Figs. 69 and 70 illustrate how one-half of the light zone gradually merges into the cytoplasm, while the other is sharply divided from it. Fig. 71 shows this area, which usually contains a large chromatin body, merging gradually into the cytoplasm. In Figs. 72 and 73 light areas are shown in the neighborhood of the nucleus and the chromidia, not sharply differentiated from the plasma. A uniform distribution of chromidia, coarse network, and light zones is shown in Figs. 74 and 75. Whether or not Figs. 65 to 68 illustrate central vacuoles, we are unable to say. But as there are various transitional pictures between these structures and the typical central vacuole formations, we have interpreted them as belonging to such a group. The sharp differentiation of the light area from the true plasma is in all probability due to the relatively slow flow of the protoplasm. In the earlier stage, where the life processes are very active and the movement of the ameba is vigorous, we do not find this to be the case, for the active plasmic flow then tends to mix the substances given off by the nucleus with the endoplasm. Later on, when the flow is retarded, these substances accumulate in a circumscribed area in the vicinity of the nucleus. This process also explains the merging of the finely reticulated structure of the protoplasm into the irregular coarsely reticulated structure in stained preparations of the large chromidial organisms. It also explains the irregular distribution of the finely reticulated structure of the small chromidial organisms in the process of formation. The light area has an intimate relation to the nucleus and chromatin. Frequently a narrow light band surrounds a nucleus rich in chromatin and chromidia (Figs. 75 and 76). This undoubtedly consists of homogenous fluid. The width of the zone is usually in direct proportion to the abundance of chromatin and inversely proportional to the plasmic flow. With abundant chromidia and slight plasmic flow, the substance tends to increase in the cytoplasm of the ameba and forms finally a central vacuole.

Inasmuch as a certain type of central vacuole (Fig. 66) with coarse network forms occasionally a new organism and again a membrane on the surface, we may assume that we have to do here with a process of regeneration, and that the central vacuole plays an important part

when conditions are unfavorable to amebic life. The chromidia may be interpreted in a similar manner. They participate in the formation of the cyst, as well as the nuclear membrane. On the other hand, they possess the character of the primitive nucleus and are able to proliferate. For that reason they may play an important part in the formation of the new nucleus, when the original nucleus degenerates.

The network of the central vacuole stains less intensely on the whole than the chromidia, and is always coarse and irregularly disposed. Frequently it becomes resorbed and disappears. In other instances it contracts in the form of rods or wedges and is changed into chromidia. In preparations stained with hematoxylin, one frequently sees chromidia stained with varying intensity.

The three substances, *i.e.* chromidia, meshwork, and the homogenous substance, stand in intimate relationship. It is not certain whether they arise from the nucleus or the cytoplasm, but it seems logical to assume, in view of the facts stated above, that they have their origin in the nucleus.

Fate of the Small Vegetative and Atypical Forms.—When external circumstances are unfavorable, the small vegetative and atypical amebæ assume a spherical shape with a doubly refractive contour. The further course of these two forms is various. Sometimes they burst suddenly, become slightly refractile, and show freed granules which finally disappear. Or they may retain the spherical shape, or divide into two equal parts, to pass finally into the cyst. Fresh preparations taken from the medium at this stage frequently exhibit this process.

The Cyst.

The development of the cystic capsule has not yet been clearly demonstrated. It appears, however, that it arises in a manner similar to the nuclear membrane and the membrane surrounding the central vacuole. Capsule formation proceeds also through the admixture of abundant nuclear substance with the plasma. Hence the nuclear substances appear to exercise a definite part in the formation of the capsule.

In nineteen examinations we found cysts containing four nuclei only on three occasions. One was a test made with red blood corpuscles

after 24 hours, another after 72 hours, and the third after 24 hours. In the last instance we were able to demonstrate numerous cysts.

The morphology of the cysts found in the culture medium (Figs. 10 and 99) corresponds with the cysts of *Entamœba tetragena* obtained from the feces of persons convalescing from amebic dysentery. On close observation, however, the latter cysts present a different constitution. The protoplasm in some organisms shows a relatively coarse network and stains but faintly with hematoxylin; in others it is finely reticular and takes hematoxylin intensively. The cysts observed by us in our experiments correspond to the latter group, while the bi- and mono-nuclear cysts resemble the first group. The multinuclear cysts found in three experiments and the binuclear cysts in another test were all observed among the small chromidial organisms with atypical nuclei. This coincides with Hartmann's finding of degenerative forms just prior to encystment in amebæ obtained from man. Our observations also call to mind the statement of Viereck that the nucleus at the time of encystment becomes indistinct owing to the formation of chromidia. On the basis of this finding we have arrived at the theory that the small chromidial organisms with atypical nuclei constitute the first stage in the formation of the tetranuclear cyst. After studying the morphology of the amebæ from this standpoint, and in particular the nuclear division, we came to the conclusion that amitotic division into four nuclei may be possible in addition to the mitotic division which has already been established.

Nuclear Division of the Vegetative Forms.—The vegetative forms have, as a rule, one or two nuclei, but never more than this number. Cell division in the typical nucleus always proceeds mitotically (Figs. 80 and 81), as described by Hartmann. The centriole divides in two, the karyosome forms a spindle between these parts, and the nuclear membrane becomes constricted in the middle. In the atypical nuclei, however, cell division varies as follows:

1. Mitotic division, as described by Hartmann for *Entamœba histolytica*.

2. The nucleus is dumb-bell-shaped and the remains of the karyosome divide in two. The centriole is not sharply differentiated (Figs. 60 and 84).

3. The nucleus without karyosome becomes constricted and dumb-bell-shaped, and divides into two equal parts (Fig. 86). On the nuclear membrane, masses of chromatin appear. This mode of division resembles amitosis, but it is impossible to say whether or not a primitive mitotic division of the nuclear membrane has taken place.

4. The compact nucleus divides simply into two equal parts (Figs. 103 and 113).

5, *a*. The nucleus elongates and forms a spindle. At each end of the two poles of the spindle is a mass of chromatin which gives rise to a nucleus (Figs. 87 and 104 to 106).

5, *b*. The abnormally elongated nucleus expands at both ends and assumes a spherical shape, giving rise to two daughter nuclei. The membrane uniting them atrophies and disappears (Figs. 83 and 89).

6. A part of the chromatin passes into the plasma and forms chromidia. The latter group themselves into a single or several spherical or crescent-shaped bodies (Figs. 98, 100, and 101). In the middle of the spherical body a vacuole appears, and surrounding it is a nuclear membrane. In the latter case a clear area appears, particularly on the concave side, and a new nucleus is formed, while the mother nucleus degenerates gradually. At times the daughter nucleus is united to the degenerating mother nucleus by a chromatin thread (Fig. 88). This formation of the nucleus is frequent shortly before encystment.

7. The nucleus extrudes into the plasma abundant chromatin bodies which condense in a circumscribed area and later form a nucleus (Figs. 92 and 93). Popoff observed this phenomenon in *Entamœba minuta*.

A similar process occurs sometimes in the small vegetative atypical forms, shortly before encystment. One nucleus has little chromatin and resembles a new formation, while the other becomes pyknotically stained and degenerates. Or the two nuclei may be unequal, one being of the usual size and somewhat degenerated, while the other is very small and contains little chromatin, like the nucleus in the tetranuclear cyst. These have probably arisen through a process of division as described under 6 and 7.

The small vegetative forms no longer exhibit motility. They become spherical, and extrude from one part a bud-like, transparent, amorphous mass of protoplasm which enlarges gradually, becoming

finally as large as the mother organism; through constriction it assumes a spherical shape. In the stained preparations the protoplasm of the mother cell is of a cloudy appearance; the nucleus is polygonal and degenerates. The daughter organism shows a few faintly stained, fine granules and a newly formed nucleus with sparse chromatin, as in *Entamœba histolytica*. In the middle of the nucleus is a very small centriole, and near it a faintly stained karyosome. In addition to constriction, division of the ameba may also occur by budding and the formation of two equal parts (Figs. 116 and 117).

Nuclear Division of the Cyst.—The cysts exhibit pictures of division similar to those of the vegetative forms.

1. The chromatin which has accumulated in a few large masses on the nuclear membrane forms from one to four small nuclei (Figs. 94, 95, 96, and 125). The daughter nuclei are found inside the enormously expanded mother nucleus (a form of central vacuole) (Fig. 118).

2. A single nucleus divides into three or four daughter nuclei (Figs. 90 and 91).

3. The substances extruded by the nucleus form four small daughter nuclei in the plasma, and the mother nucleus degenerates gradualiy. The daughter nuclei are seen outside the degenerating mother cell (Fig. 99).

We may conclude, therefore, that the outer chromatin in common with the centriole possesses the power of regenerating a nucleus and of forming chromidia, and that the typical vegetative form and its cysts show mitotic cell division, while the atypical forms and their cysts reproduce by amitotic or primitive mitotic division. Figs. 108 to 112 illustrate cysts, taken from the feces of patients convalescing from amebic dysentery. It is clear that the cyst is that of the dysentery ameba, since the patients did not harbor the *coli* organism, or a cyst of any other type of ameba. Four daughter cells are visible and a large degenerating nucleus. This picture is frequently found among the tetranuclear cysts. The degree of degeneration of the large nucleus varies. The process cannot be explained solely on the basis of mitotic division. Fig. 99 illustrates a cyst found in the culture medium.

CONCLUSIONS.

Dysentery amebæ may live for 72 hours *in vitro*.

The transition of the large vegetative forms into the cyst may be observed *in vitro*.

The large vegetative ameba always passes into an intermediate stage before assuming the small vegetative form.

The atypical forms are dysentery amebæ with abnormal nuclei and cytoplasm. When circumstances are unfavorable encystment takes place. Under favorable conditions, on the other hand, they may again change into typical forms. The majority of the "degenerative" forms of Hartmann cannot be so classed. According to our observation, they represent forms which have been described here.

Hartmann's *Entamœba histolytica* is really a typical transitional form of *Entamœba tetragena*.

The origin and significance of the central vacuole have been elucidated.

Nuclear division of the dysentery ameba proceeds in three ways. The typical forms divide on the whole mitotically, and the atypical amitotically or in a primitively mitotic manner.

In conclusion we desire to express our obligation to Professor S. Ogawa for studying our preparations, and to Professor R. Inada and Professor Y. Ido for the stimulus and supervision which they have given to our work.

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EXPLANATION OF PLATES.

Figs. 14, 15, 20, *b*, 52, 53, and 54 represent living specimens; the remaining figures are from preparations stained with hematoxylin, according to the methods of Heidenhain and Delafield.

PLATE 30.

FIGS. 1 to 10. Amebæ with typical nucleus.
FIG. 1. Large vegetative form.
FIGS. 2 to 4. Intermediate form.
FIGS. 5 and 6. Small vegetative form.
FIGS. 7 and 8. Small chromidial organisms.
FIG. 9. Mononuclear cyst.
FIG. 10. Tetranuclear cyst.
FIG. 11. Ameba in another culture medium, or at higher temperature.
FIG. 12. Small chromidial organism with large vacuole.
FIG. 13. Mononuclear cyst with central vacuole.

PLATE 31.

FIGS. 14 to 23. Various transitional reduction phases of amebæ.
FIG. 14. Passage of excretory matter from posterior wall of ameba.
FIG. 15. The elongated mononuclear ameba becomes constricted in the middle, and divides into two parts.
FIG. 16. The elongated mononuclear ameba becomes constricted in the middle, and divides into two parts.
FIGS. 17 to 22. Amebæ with buds.

PLATE 32.

FIGS. 23 to 27. Fusion of two amebæ.
FIG. 79. Small vegetative forms, united by a stem.

PLATE 33.

FIGS. 28 to 49. Nuclei, showing cyclic changes of karyosome.
FIGS. 28 to 31. Nuclei of large vegetative forms.
FIGS. 32 to 49. Nuclei of transitional and small vegetative forms.
FIGS. 50 and 51. Nuclei of atypical forms.

PLATE 34.

FIG. 52. Transitional form.
FIG. 53. Small vegetative form, showing pseudopodium.
FIG. 54. Small vegetative form at rest.
FIGS. 55 to 59. Large atypical forms.

FIGS. 60 to 62. Small atypical forms.

FIG. 63. Small vegetative form with two nuclei and vacuole. One nucleus is typical, the other degenerated.

PLATE 35.

FIG. 64. Cyst; endogenous nuclear formation.

FIG. 65. Ameba with endogenous nucleus.

FIG. 66. An organism freed by tearing away of plasma.

FIG. 67. Small atypical form with central vacuole.

FIG. 68. Small atypical form with central vacuole holding two nuclei, upper degenerated, lower newly formed.

FIGS. 69 to 72. Small vegetative forms with normal central vacuole.

FIGS. 73 to 76. Large chromidial organisms.

FIGS. 77 and 78. Small chromidial organisms.

PLATE 36.

FIGS. 80 to 99. Various nuclear divisions and cyst formations.

FIG. 80. Mononuclear, small vegetative form. Mitosis.

FIG. 81. Mononuclear cyst. Mitosis.

FIGS. 82 to 84. Small atypical forms. Primitive mitosis.

FIG. 85. Binuclear, small vegetative form, shortly after division.

FIG. 86. Small atypical form. Amitosis.

FIG. 87. Binuclear atypical form. One nucleus is dividing. The other, a daughter nucleus, is formed from the substance of the mother nucleus.

FIG. 88. Small atypical form. The daughter nucleus is united to the degenerating mother nucleus by chromatin threads.

FIG. 89. Small atypical form. The elongated nucleus enlarges spherically at both ends, giving rise to two daughter nuclei.

FIGS. 90 and 91. Multiple nuclear division of small atypical forms, shortly before encystment.

FIGS. 92 and 93. Small vegetative forms. Chromatin condensed in circumscribed area, which later forms a nucleus.

FIGS. 94 to 97. Encystment of small atypical forms. Multiple nuclear division. Outer chromatin later forms four daughter nuclei.

FIG. 98. Cyst with three nuclei; the two nuclei to the left are newly formed; the other is degenerating.

FIG. 99. Tetranuclear cyst with degenerating mother nucleus.

PLATE 37.

FIGS. 100 to 117. Various nuclear divisions and cyst formations.

FIGS. 100 and 101. Small vegetative forms with mother nucleus and daughter nucleus arising from the chromatin of the former.

FIG. 102. Small atypical form with nucleus showing two constricted masses of chromatin.

FIG. 103. Small vegetative form with three compact nuclei.

FIGS. 104 to 107. Small atypical forms. Nuclear formation from outer chromatin.

FIGS. 108 to 112. Cysts obtained from feces of convalescents, amebic dysentery.

FIG. 108. Cyst with one small and one large nucleus.

FIGS. 109 to 112. Cysts with four small nuclei and the remnant of a large nucleus.

FIG. 113. Small atypical form with two compact nuclei.

FIG. 114. Small atypical form with two constricted, compact nuclei.

FIG. 115. Small atypical form with one large, newly formed nucleus and one degenerating nucleus.

FIGS. 116 and 117. Budding, dysentery amebæ.

FIG. 118. The same as Fig. 111.

PLATE 38.

FIG. 119. Large vegetative form. × 950.

FIG. 120. Constriction of transitional form. × 950.

PLATE 39.

FIG. 121. Transitional form with bud. × 950.

FIG. 122. Binuclear large chromidial form. × 950.

PLATE 40.

FIG. 123. Small vegetative form. × 950.

FIG. 124. Small chromidial forms. The individuals are united by protoplasmic stems. × 950.

FIG. 125. Encystment, atypical form. Multiple nuclear division. × 950.

PLATE 41.

FIG. 126. Tetranuclear cysts. × 950.

FIG. 127. The same as Fig. 67. × 950.

PLATE 42.

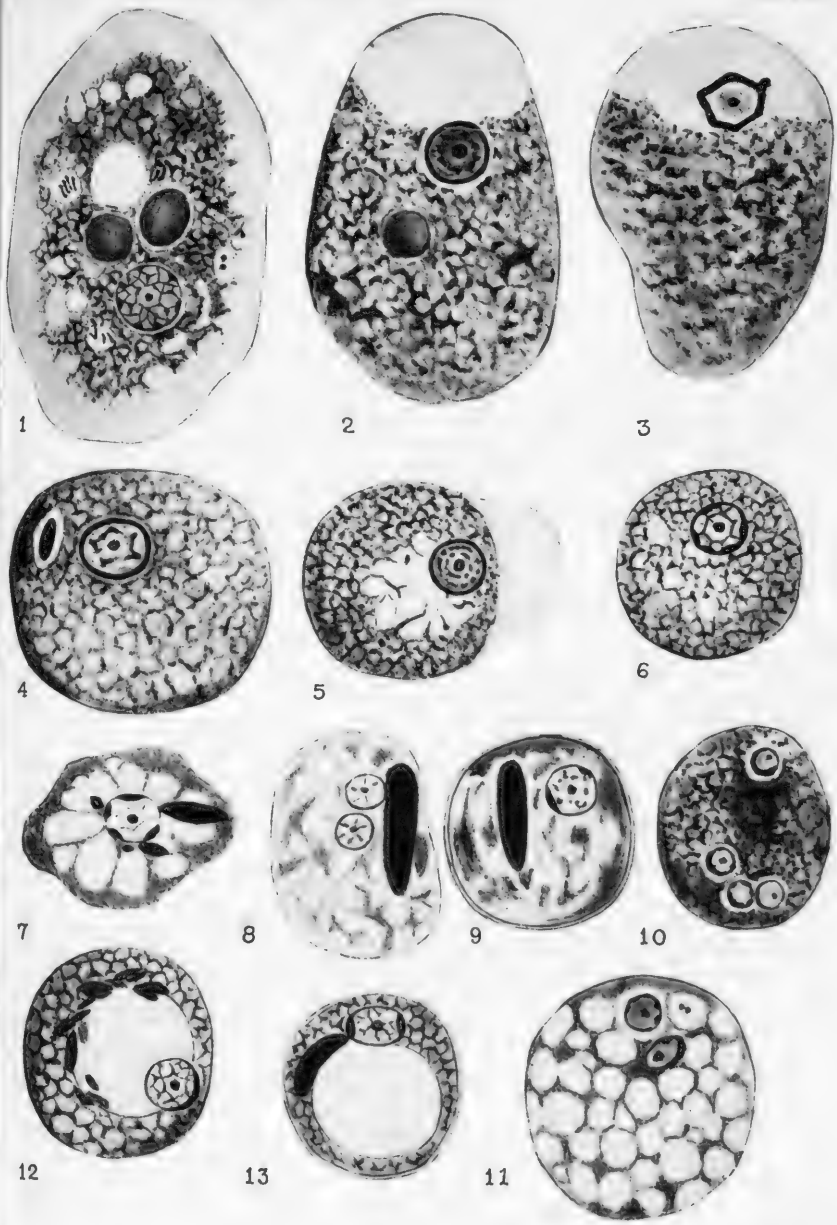
FIG. 128. Large chromidial forms and large atypical forms. × 950.

FIG. 129. Small atypical forms. Many individuals united by thin protoplasmic stems. × 950.

PLATE 43.

FIG. 130. Transitional forms. × 750.

FIG. 131. Small vegetative form. Mitosis. × 750.



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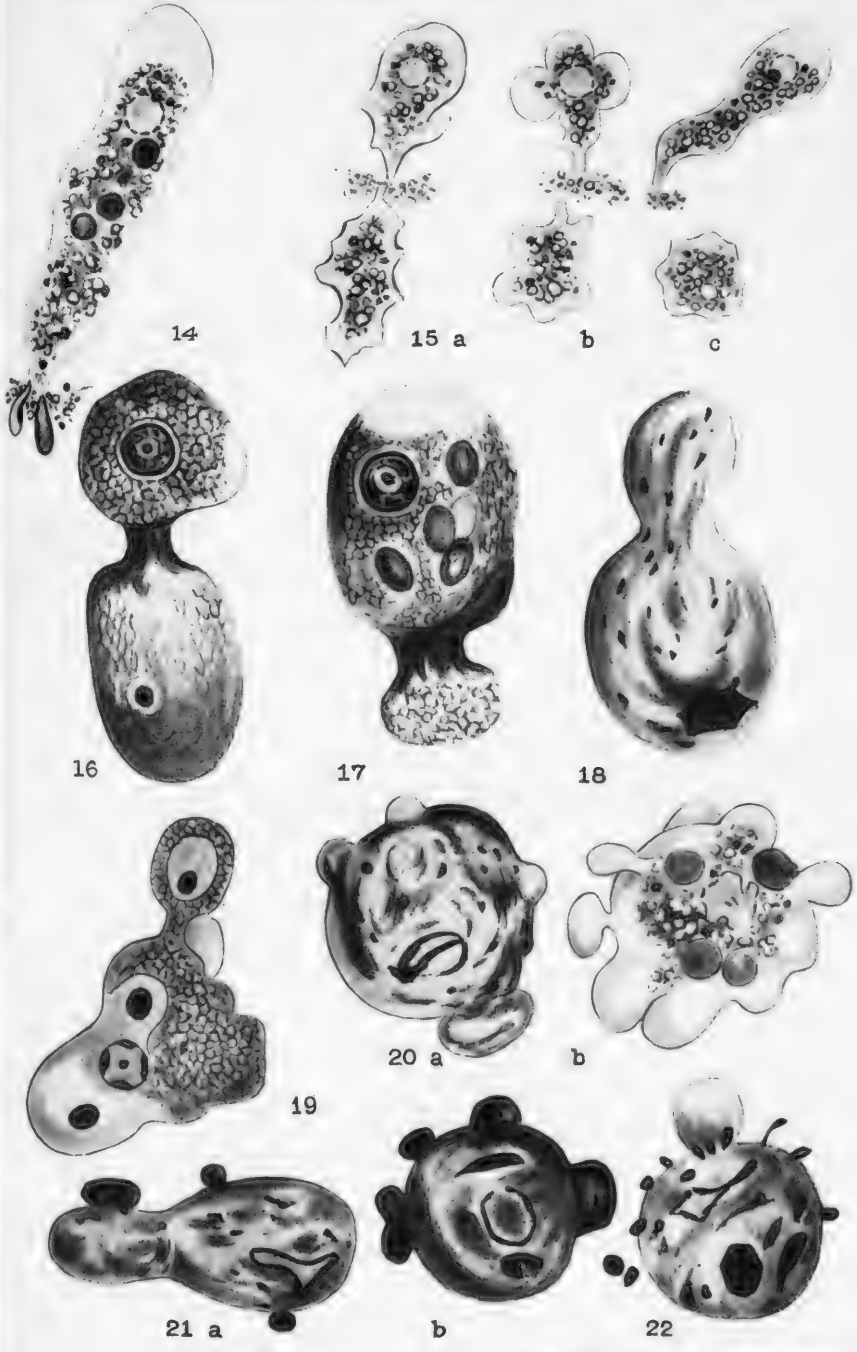

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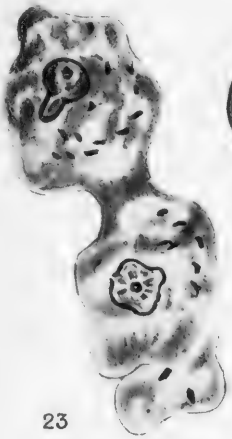

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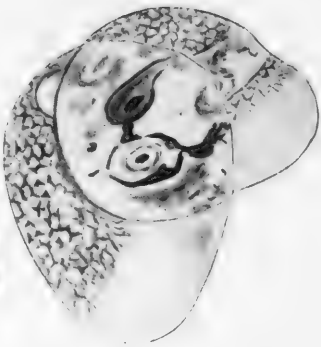

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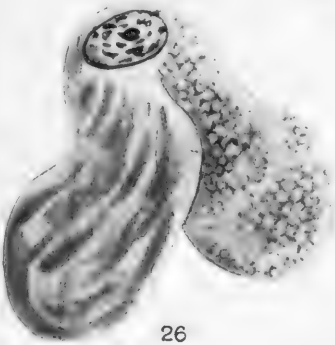

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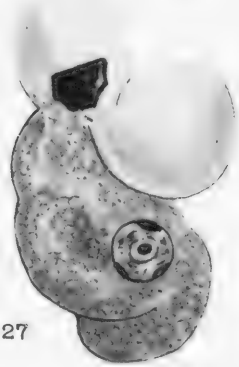

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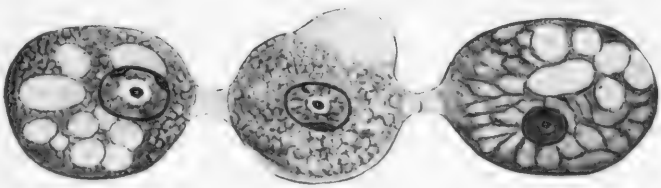

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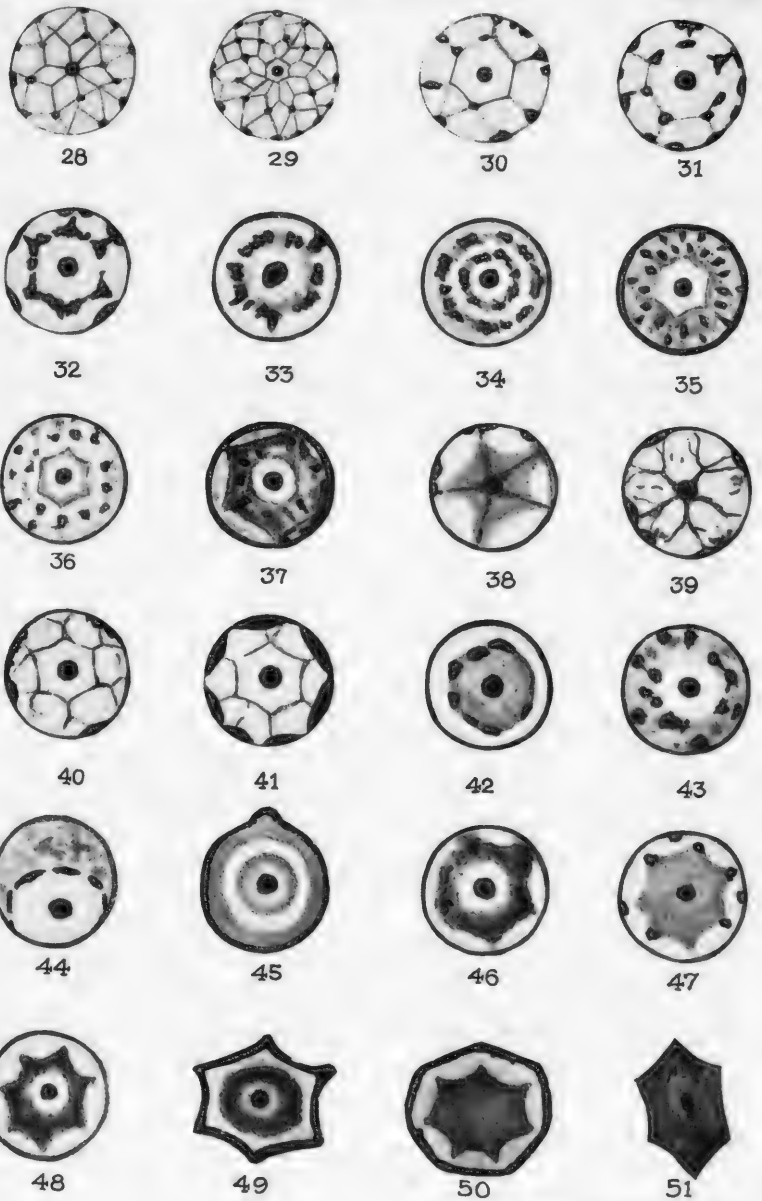
(Yoshida: Encystment of dysentery amebæ.)



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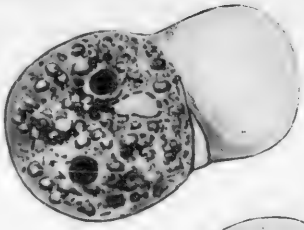

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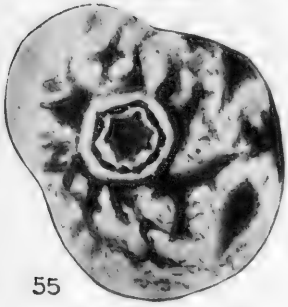

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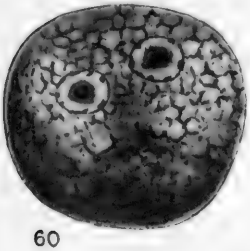

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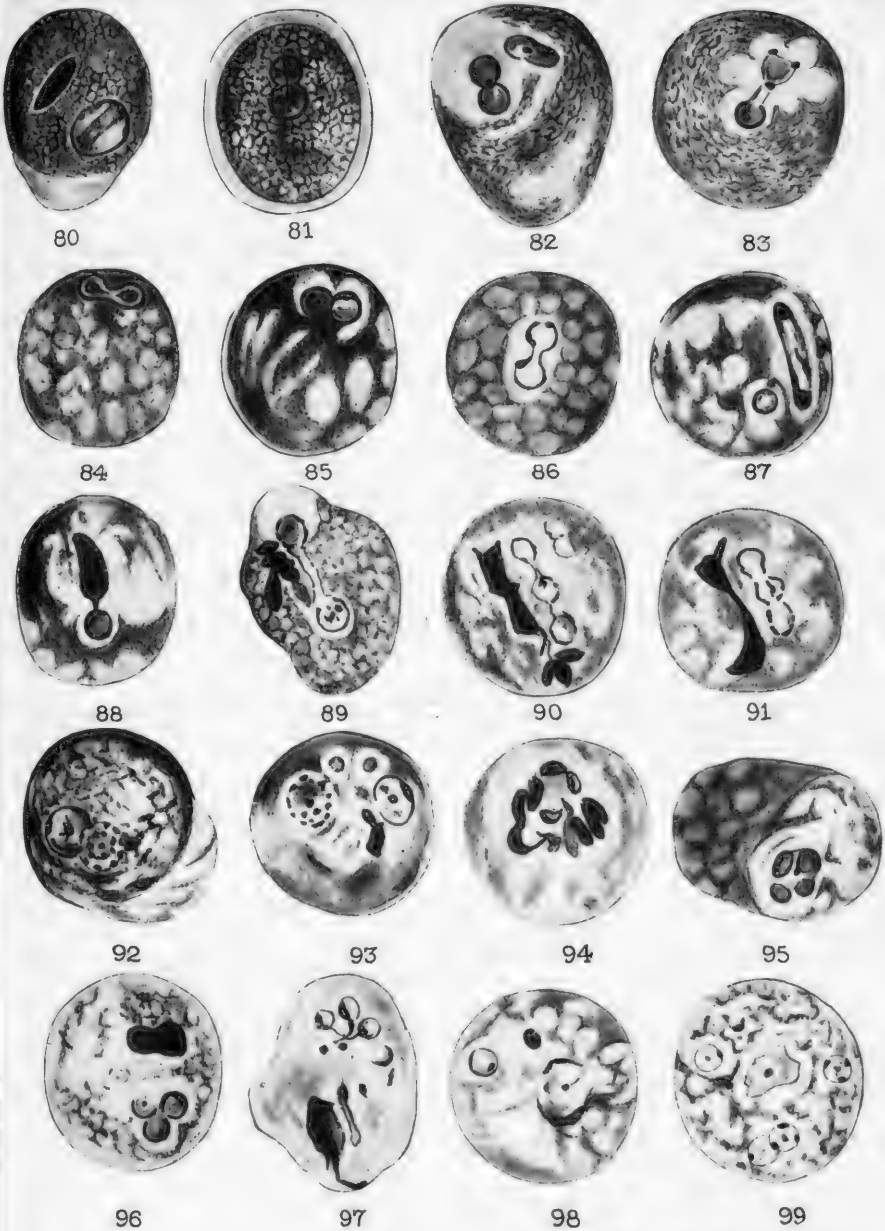
(Yoshida: Encystment of dysentery amebæ.)



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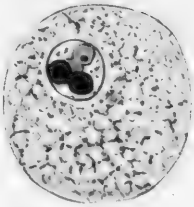
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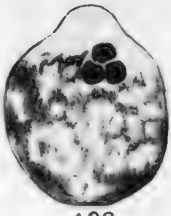
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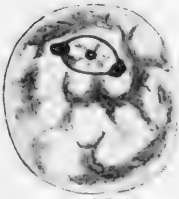
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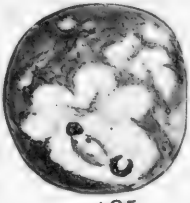
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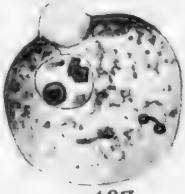
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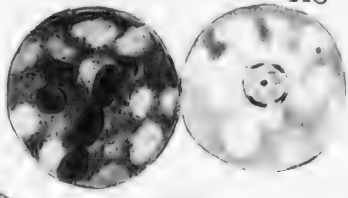
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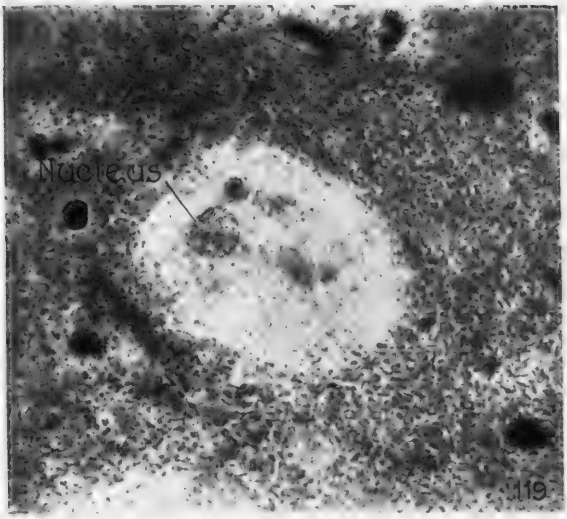
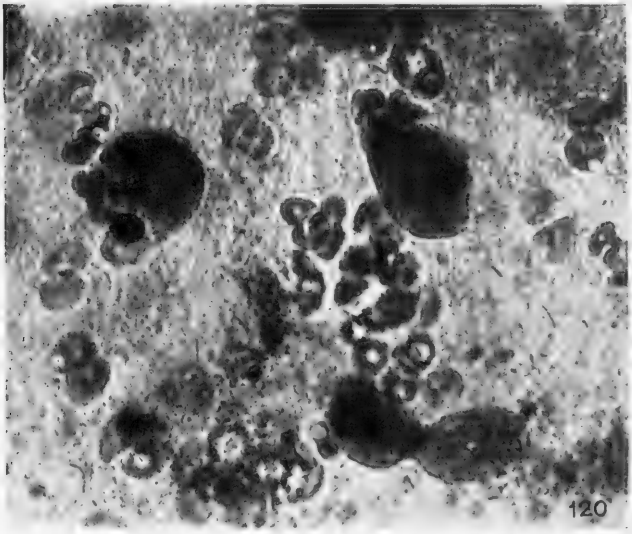
(Yoshida: Encystment of dysentery amebæ.)





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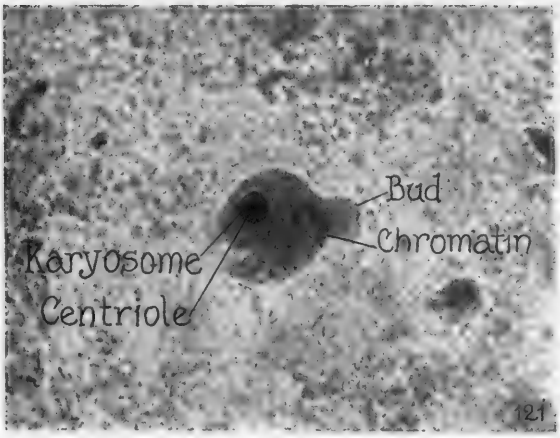

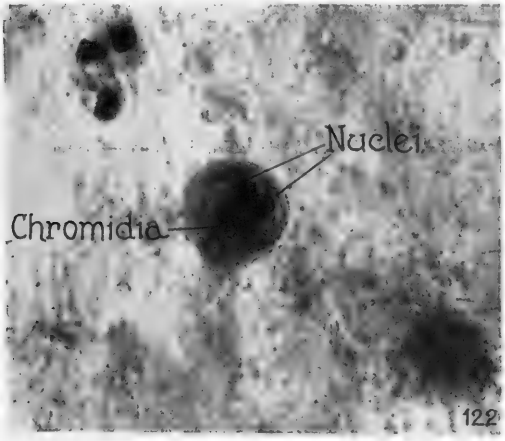

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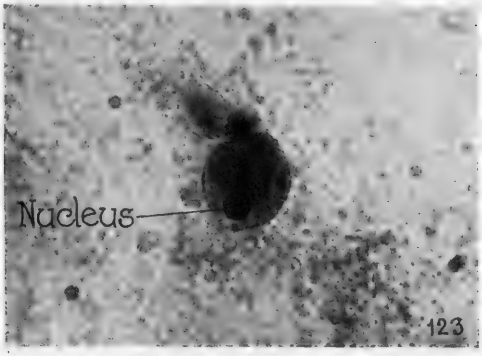

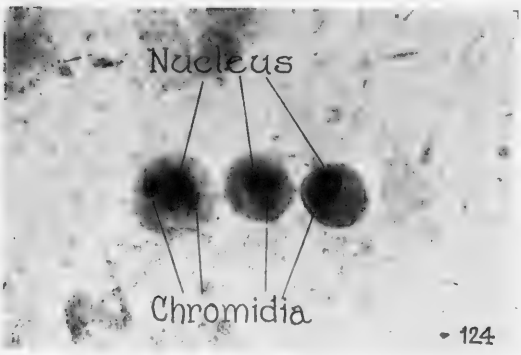

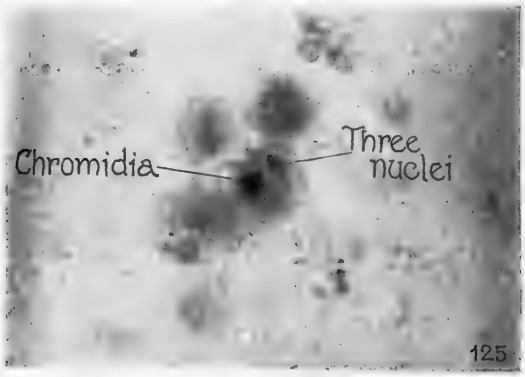

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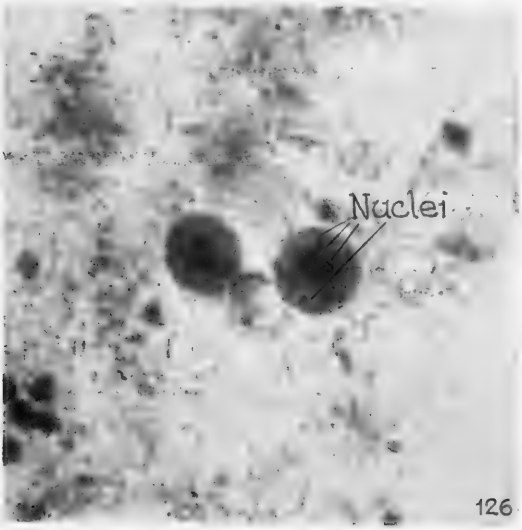
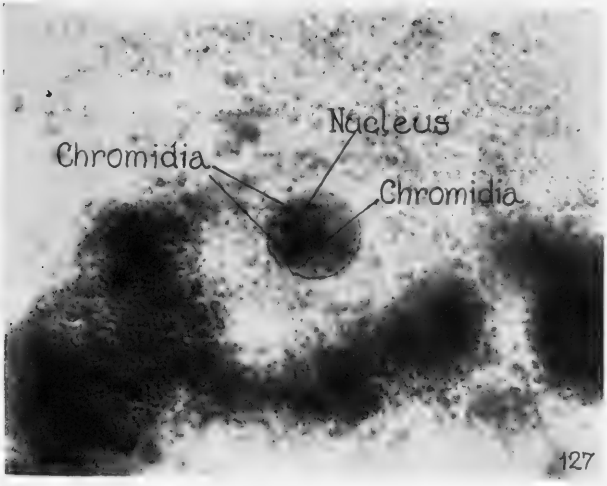
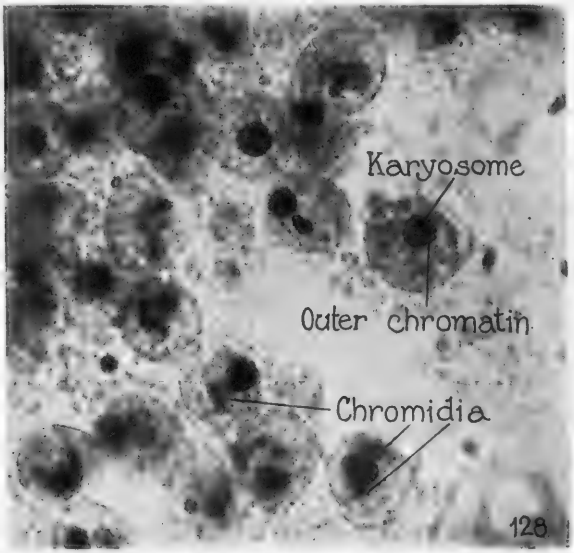
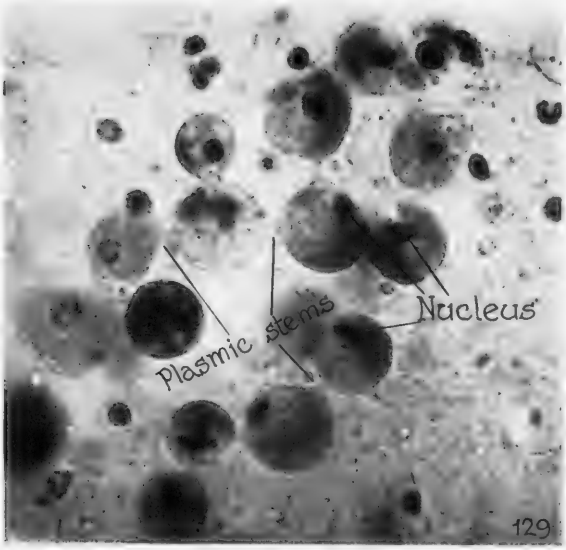




(Yoshida: Encystment of dysentery amebæ)





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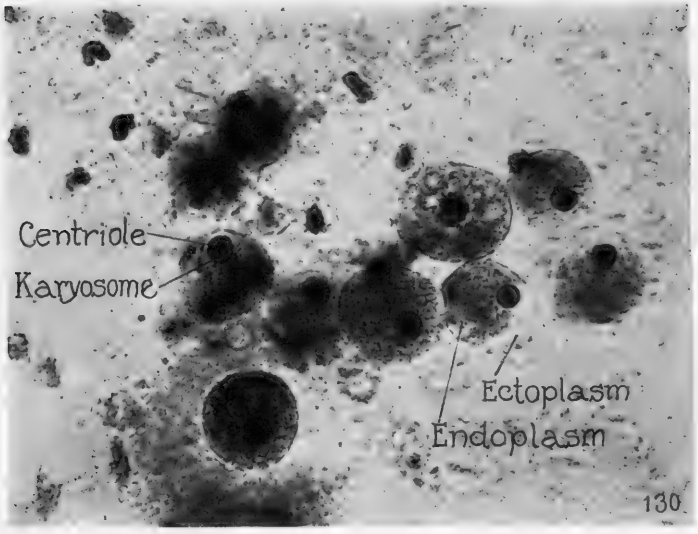

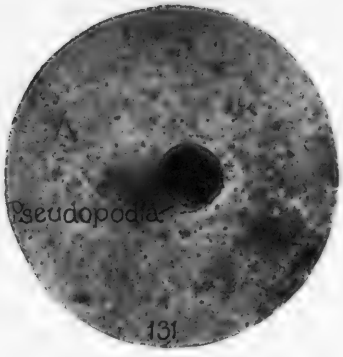

(Yoshida: Encystment of dysentery amebæ.)

A NEW NON-PATHOGENIC TETRAGENOUS AMEBA. I.

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PLATES 44 AND 45.

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In the study of the amebæ of dysentery we have discovered in the feces of healthy persons a new type of ameba, which, like the dysentery ameba, forms a cyst containing four daughter nuclei, but which differs from it in biological and morphological properties. Experiments made upon animals and clinical findings have demonstrated the non-pathogenicity of the new organism.

The ameba to be described has been observed by the author in six cases; *i.e.*, in five persons with a normal condition of the intestines and in one having a chronic catarrh of the large intestine, not dysenteric in character. In every instance the vegetative form of the ameba was found in the saline (Carlsbad salt) diarrheal stools and the cyst in the formed stools. The persons failed to show any symptoms of amebic dysentery, even after long purging; on discontinuing purgation they evacuated normal dejecta and showed no manifestations of illness.

Morphological.

As fresh stools as possible were used for the study. The vegetative forms were examined microscopically directly after defecation, in a hanging drop preparation, at a temperature of 30–37°C. Sublimate alcohol and iron hematoxylin were used for fixation and staining.

Like the dysenteric amebæ, these organisms occur in three forms: (1) large vegetative form; (2) small vegetative form; (3) cyst.

Large Vegetative Form.—This form is consistently present in every part of the naturally evacuated diarrheal feces or those obtained after purgation. The active organisms can be observed readily in a hanging drop preparation from a fluid or watery stool. Their move-

ment resembles that of the dysenteric ameba, although it is on the whole slower. The changes in the shape of the organisms and the plasmic flow proceed rather slowly. One has the impression of a snail dragging its shell. The ectoplasm and endoplasm are not easily distinguished when the organism is at rest, but can be observed during movement, though not so distinctly as in *Entamœba tetragena* Viereck. A few hours after defecation this movement is discontinued. The amebæ have then become spherical in shape, and rarely extend pseudopodia. But if a preparation of this kind is returned to the thermostat, a relatively lively motion may be observed after a few hours.

The cytoplasm is, as a rule, coarsely meshed and of a tough, compact structure. It has slightly refractile properties. No blood corpuscles are found in the cytoplasm, but various bacteria or food remnants may be present. Food vacuoles can be plainly seen, but no contractile vacuoles. In the food vacuoles one frequently finds numerous bacteria, particularly staphylococci, which show vigorous molecular movement. This circumstance, observed rarely in the dysentery ameba, is present in *Entamœba coli.* In accordance with the view of Hartmann that the kind of food remains found in the protoplasm may be considered a characteristic in differentiating the non-pathogenic from the pathogenic amebæ, we desire to advance the finding of cocci as a characteristic of the ameba under discussion. The ectoplasm is of glassy transparency, light-refracting, but not as markedly so as in the case of the dysentery ameba.

The nucleus resembles a vesicular sphere and lies eccentrically. It is plainly visible even when the organism is at rest. The nuclear membrane is thick and tough, though occasionally it is smooth and thin as in the dysentery ameba. Surrounding the nucleus a narrow, light area may be observed. In this respect the nucleus resembles that of *Entamœba coli.* As in the latter, the nucleus also contains numerous, markedly refractile, fine granules, particularly along the wall. The nuclear membrane may be absent, in which case a chain of the refractile granules forms a ring about the nucleus. In the flow of the endoplasm the nucleus always retains its globular form.

In the stained preparations the cytoplasm is not homogenous but is in part coarsely meshed, with a structure resembling that of *Enta-*

mœba coli or the smaller form of dysentery ameba. The cytoplasm frequently contains brownish food residues and darkly stained bacteria. When the latter are located within the food vacuoles, they may be mistaken for the dividing nucleus or the newly formed chromidia of the enlarged nucleus. If the organisms are fixed and stained while motile, one may obtain various pseudopodia stained light brown. Otherwise the organism appears spherical or oval; the ectoplasm is indistinct.

The size of the large vegetative ameba is 15 to 19μ, the largest measuring 25μ; the nucleus is from 1.5 to 4μ. The chromatin outside the nucleus consists of coarse granules which adhere to the nuclear membrane. The nucleus contains abundant chromatin, which may be distributed diversely in the fine linear alveolar tissue, in small or in a few large particles. In the center lies a relatively large and distinct centriole as in the dysentery organism. Surrounding the centriole one is often able to observe a narrow, clear area. The nucleus does not show typical cyclic changes such as Hartmann and Werner observed in the dysenteric ameba.

Small Vegetative Form.—In contrast to the dysentery ameba, it is difficult to distinguish the small form of the non-pathogenic ameba from the large vegetative form. The large form is found in diarrheal stools; when, however, the feces increase in consistency the smaller forms appear in addition to cysts. They are minute in size, measuring usually 6 to 13μ, the largest found being 19μ (Figs. 7 and 8).

As in other amebæ, we may regard the small form as the first stage of the cystic transformation, even though it may persist for some time. In this stage the food taken up by the cytoplasm has been digested or extruded, so that there is but little foreign material present. Some of the specimens show a few bacteria in the vacuoles (Fig. 8). The cytoplasm is coarsely meshed. The ectoplasm is clear and transparent, and recognizable only when the organism is motile. Because of their greater refractibility, one may observe these smaller forms in a fresh preparation under the low power lens. The movement is much slower than in the larger form of ameba, and it is possible to distinguish readily the extrusion of the pseudopodia, the plasmic flow, and locomotion.

The nucleus of the small form of the non-pathogenic tetragenous ameba shows in general the characteristics of the small form of *Entamœba tetragena* Viereck, but with a little experience a number of distinguishing characteristics are brought to light. The nucleus, as in other entamebæ, is vesicular. Some specimens, owing to the coarse granules, appear irregularly formed. The nuclear membrane is thick, and the peripheral chromatin abundant, sometimes coarse and irregular, again occurring in a few large masses. In the center of the nucleus lies a relatively large centriole, which occasionally presents the picture of division. The cyclic phenomena described by Hartmann for *Entamœba tetragena* Viereck do not take place. One frequently sees an organism with two nuclei, but never one with four.

Cyst.—The cyst is usually found in the formed or soft stools, and particularly after diarrhea. As a rule, this type of ameba forms cysts abundantly, while they occur but sparingly with the dysenteric ameba. When the non-pathogenic ameba is found in the feces, it is always possible to find numerous cysts also.

The cyst, which is highly refractile, is as a rule spherical, at times oval. In the process of transformation of the vegetative into the cystic form the volume of the cytoplasm is first reduced by an expulsion of foreign matter and fluids. It is thus rendered clear and transparent, and the meshy structure becomes distinct. The nucleus appears somewhat swollen and becomes rich in chromatin which accumulates in the center and along the outer periphery. A number of chromidia are seen in the cytoplasm. Simultaneously with these changes in the nucleus, appears a clear space, like a vacuole, in the cytoplasm (Fig. 11) which gradually increases in size (Fig. 12). In the stained preparations the nuclear content is seen to become gradually rich in chromatin, so that the large and small chromatin bodies surrounding the karyosome become visible. The chromatin along the nuclear membrane forms in masses, some of which proceed from the nucleus into the cytoplasm where they proliferate. These chromidia are located along the periphery or irregularly around central vacuoles (Fig. 13). One sees frequently that the chromidia and the nucleus are pressed against the nuclear wall because of the enormous dilatation of the central vacuole. In the course of time the elongated nucleus is constricted into two nuclei. In this process the central vacuole

decreases gradually in size and finally disappears, and the cytoplasm which previously had been crowded toward the periphery, now becomes voluminous again. The number of chromidia is also decreased. Some remain occasionally in the cytoplasm in the form of chromatin particles. In this stage it is easy to distinguish the cystic membrane.

The mature cyst contains four nuclei. Its diameter in fixed preparations averages from 6 to 12μ, the size of the daughter nuclei being 1.2 to 2.5μ. As Kuenen and Swellengrebel have described for the dysentery ameba, we can also trace in the cyst of the ameba under discussion a kind of supplementary nucleus, smaller than the normal nucleus. It has the appearance of spherical bits of chromatin. As a rule, the supplementary nucleus does not stain well, so that it can be distinguished readily from the normal nucleus.

The fact that the chromidia attain their maximal growth simultaneously with the appearance of the vacuoles, and that they disappear at the same time, points to the conclusion of their genetic relation.

Mitosis and Reproduction.—As Schaudinn has described for *Entamœba coli*, the reproduction of the vegetative forms of the non-pathogenic tetragenous ameba also proceeds by simple division. We often see the large and small vegetative forms of the ameba harboring two nuclei (Figs. 3 and 9), which arise in the following manner. The original nucleus becomes enlarged to about twice its size. On the one side an increase in chromatin bodies takes place, while on the other the central karyosome divides into two parts which are united sometimes by a fine filament. The enlarged nucleus gradually becomes elongated, and is then constricted in the middle and divides by amitosis. The cytoplasm follows the same procedure. A typical spindle is rarely seen.

This simple cell division must be considered as the usual mode of reproduction of the non-pathogenic ameba. The forms having two nuclei are most numerous in diarrheal stools following purgation. From this we may conclude that this division plays a large part in the rapid proliferation of the ameba. Kuenen and Swellengrebel also observed this division which they have described minutely. Our findings are in accord with theirs.

In addition we have observed quaternary division, such as Schaudinn, Hartmann, and others have found in *Entamœba coli* and described as schizogenesis. A nucleus is found to divide first into two, and then into four parts (Fig. 4). In the vegetative form we were unable to observe this process. After a certain length of time we have found four small amebæ with four nuclei (Fig. 5) arising from the one organism. This process of division is found in amebæ obtained from fluid stools, but as a rule less frequently than the division which produces two amebæ. In experiments conducted previously with the dysentery ameba, we found frequently in the intestinal tissues of animals killed when the dysentery was at its height, amebæ containing two nuclei, but rarely organisms harboring four nuclei.

The amebæ produced by simple division or schizogenesis are on the whole very small, measuring only 6 to 11μ. They have a relatively large nucleus which is plainly visible in fresh preparations. In the center is a nucleolus half or two-thirds as large as the nucleus; it stains intensely with hematoxylin. The nuclear membrane is thin and smooth, and not rich in chromatin. Between the nuclear membrane and the karyosome no chromatin is visible. The cytoplasm is of coarse, mesh-like structure. The endoplasm cannot be readily distinguished from the ectoplasm. No foreign substances are visible in the cell substance. The young amebæ are very clear and transparent in fresh preparations.

The processes of encystment and cell changes are somewhat complex, but coincide in several points with those of *Entamœba coli.* The ameba decreases in size, retaining, however, a meshy structure of the plasma, as in the vegetative form. It now contains no foreign material, so that at this stage the organism appears perfectly transparent. The refractile nucleus is easily recognizable. The nucleus then increases its chromatin, which accumulates in the center and along the periphery. In the cytoplasm one sees variously formed chromidia. Upon the expulsion of the chromidia, a vacuole appears in the middle of the cell substance (Fig. 11), which increases in the course of time and comprises finally the larger part of the cell substance. The cytoplasm and the chromidia are crowded into the small area along the periphery. This causes the nucleus to assume an oval shape; the centriole divides into two parts which are

gradually drawn toward the two poles. One often sees divided centrioles still attached by a fine thread. Typical spindles are often found. Upon the completion of nuclear division there is a decrease in the size of the central vacuole which finally disappears and the cytoplasm again becomes voluminous (Fig. 17). The chromidia also decrease in number, although they may remain for a long time as chromatin particles. In fresh preparations the cytoplasm is at this stage clear and transparent. The cystic membrane is distinct, appears double, and is markedly refractile. The two freed nuclei lie side by side, but isolated by chromidia. The mass of chromidia is larger than in other forms of amebæ (Figs. 19 to 22). The two mature nuclei again divide amitotically, and we have as a result a typical cyst containing four nuclei. The four daughter cells within the cyst are isolated. Frequently one finds but few chromidia within the cyst (Figs. 39 to 42).

In addition to the manner of division described above, one may observe still another form. During the development of the cyst we see a somewhat swollen nucleus with a membrane having but little chromatin and having adherent to it a number of chromatin particles which stain intensely black with hematoxylin. This picture reminds one of Hartmann's degenerated form of dysentery ameba. After the disappearance of the nuclear membrane these chromatin particles lie together in the cytoplasm. In the end every chromatin particle is found to be a nucleus, measuring 1 to 1.5μ, smaller in size than that formed by division (Figs. 23 to 26). The centriole is often not visible. According to the above description we must assume a somewhat modified view respecting the origin of the daughter nuclei. The four isolated daughter nuclei originate from the mother nucleus after the disappearance of the nuclear membrane.

Another mode of division is frequently observed. The abundant chromatin bodies which have been developed in the nucleus wander out into the plasma, leaving but little chromatin in the mother nucleus which is finally resorbed in the plasma. The cast off, kidney-shaped chromidia then undergo change, and in due time form a nucleus. Surrounding the two chromidia is seen a narrow, clear zone (Figs. 27 to 30).

Remarks on Individuals Harboring the Non-Pathogenic Ameba.

After having had amebic dysentery the patient often evacuates cysts and has numerous relapses. It was conceivable that the organisms which we observed represented the cystic form of the typical dysentery ameba, and in fact we at first assumed this to be the case. We waited long for relapses of dysentery to appear, but notwithstanding the lengthy observation—8 months in one case—and the use of purgatives, our patients never developed symptoms of amebic dysentery. Repeated examinations of the rectal region by proctoscope failed to show pathological conditions. In only one instance did we observe slight catarrhal changes on the mucous membrane of the large intestine, but no inflammation or scars.

In our study of amebic dysentery we found that sixteen out of seventeen persons after convalescence become cyst carriers, and after the adminstration of purgatives often experience a recurrence of the disease. With the non-pathogenic tetragenous ameba the result is different. The patients failed to develop objective or subjective symptoms after repeated purgation.

Animal Experiments.

Viereck discovered in non-dysenteric stools amebæ which develop tetranuclear cysts. Unfortunately he did not supplement his studies on this point by further publications. Russage believed the tetragenous ameba to be a form of the *coli* ameba, and denied its pathogenicity. Whether or not the tetranuclear cysts observed by Viereck and Russage are identical with the cyst of the tetragenous ameba described here, cannot be definitely affirmed, since animal experiments are not reported in the writings of Viereck and Russage. On the other hand, we have conducted such experiments, and the innocuous character of our ameba can, therefore, be demonstrated not only clinically but also experimentally with paralleled observations on the cysts of amebic dysentery.

Authors are divided on the question of the pathogenicity of the dysentery ameba. Kartulis, Juergens, Hartmann, Werner, and others claim to have proved its pathogenicity by experiments upon various animals, while a number of other investigators, such as Grassi, Cunningham, and Röhmer have denied this point. In a former study of experimental amebic dysentery in young cats, we introduced the dysentery amebæ *per anum* and the cysts *per os*. Of 53 animals receiving the organism *per anum* 91 per cent proved positive; of 12 receiving them *per os*, 50 per cent were positive. The infected cats showed the typical symptoms of dysentery. The stools always contained bloody mucus and numerous amebæ

typical of that disease. Young cats of 400 to 600 gm. body weight were found to be most suitable for these experiments.

In a similar manner the author has conducted experiments with the new tetragenous ameba on twenty-three young cats of 400 to 600 gm. body weight. Of these, ten animals received the vegetative forms *per anum*, and thirteen animals the cysts *per os*. The feces used for inoculation came from three cases. In two of the animals receiving the amebæ *per anum*, 4 days after injection we found the vegetative forms in the soft stools; these findings continued for about 4 days. Then, however, the vegetative forms disappeared gradually from the stools, without being replaced by the cysts. During the whole course of the experiment the animals showed no symptoms of dysentery, but remained well. After 10 days they were killed and autopsied. Nowhere in the intestinal mucosa did we find a dysenteric focus, and there were no amebæ present in the tissues. Three of the other eight experimental animals received later several injections of the vegetative forms *per anum*. No evidence of infection could be found. The animals continued in a healthy condition.

In five of the thirteen animals fed with cysts, a few days later a small number of cysts was found in the feces. Only one of the cats evacuated after 6 days sluggishly motile vegetative forms, which, however, disappeared a few days later.

The amebæ discharged by the cats are in general morphologically identical with those obtained from human feces. They are from 12 to 14μ in size. When at rest, it is difficult to distinguish between endoplasm and ectoplasm. The cytoplasm is coarse meshed and contains little foreign material. The nucleus measures usually 1.5μ in diameter. In the center is a relatively large centriole rich in chromatin. The movements and the plasmic flow of the organisms are very slow.

As a method of enrichment for the amebic cysts, we employed the antiformin or antiformin-ether method, as well as the Ujihara method. We often found several cysts that were injured. For that reason we used, as in the infection experiments with the cysts of the dysenteric ameba, a 0.6 per cent saline solution as an enriching medium. The fecal mass containing the cysts was dissolved in the salt solution,

and filtered through double gauze. The filtrate was centrifugalized and the sediment washed several times with the salt solution, until it became relatively clean and free from fecal odor. A large number of uninjured cysts may be obtained by this procedure. In earlier experiments we had found that the cysts washed with tap water also proved good infective material for cats.

Morphological Differentiation of the Non-Pathogenic Ameba.

Entamœba tetragena Viereck.—Viereck, Hartmann, Werner, Akashi, Whitmore, Darling, and Kuenen and Swellengrebel have described this ameba in detail. The size of the large, vegetative forms is from 20 to 40μ, the largest being 60μ, while the smallest vegetative forms measure usually 20 to 25μ. The specimens of our ameba are on the whole smaller than the dysentery ameba, the largest averaging from 17 to 25μ, and the smaller forms from 6 to 19μ. In contrast to the dysentery ameba, the motility of our organism appears sluggish, and the ectoplasm can be distinguished from the endoplasm only during movement. The plasmic flow is very slow and not smooth. Red blood corpuscles are never found in the cytoplasm. The nuclei of the dysentery ameba and the non-pathogenic tetragenous ameba are much alike, but with sufficient experience a number of characteristic differences can be detected, as have already been described. We desire here merely to emphasize that in differentiation one must have in mind the entire structure of the organism.

It is difficult to distinguish the cysts of the two types of amebæ, and for this reason it is better to base judgment upon the vegetative forms than upon the cysts. The cysts of the dysentery ameba measure on the average 11 to 14μ in diameter, those of the non-pathogenic tetragenous ameba 8 to 12μ. Craig states that the cyst of *histolytica* has numerous chromidia, while the *coli* cyst harbors none; he differentiates the types of ameba on this basis. We do not agree entirely with this view, but it is certain that the chromidia in the non-pathogenic tetragenous ameba are smaller and fewer in number.

Entamœba coli Lösch emend. Schaudinn.—The vegetative forms of the *coli* and the non-pathogenic amebæ have the following points of resemblance. (1) Both are non-pathogenic for man and animals,

and may become parasitic in the normal human intestine. (2) The morphological characteristics, particularly the type of movement, the differentiation of the ectoplasm, and the structure of the nucleus, are alike up to a certain point. The two types of ameba can be readily distinguished, however, in their cysts. The cyst of *Entamœba coli* measures from 14 to 19μ in diameter; according to Kuenen and Swellengrebel, it measures 16 to 25μ. Hence this cyst is much larger than that of the non-pathogenic organism. Mature *coli* cysts contain always eight daughter nuclei. The non-pathogenic ameba forms a cyst with four daughter nuclei, never eight.

Of other amebæ—such as *Entamœba minuta, hartmanni, williamsi, butschli,* and *piteti,*— *Entamœba hartmanni* possesses the greatest similarity to our non-pathogenic ameba, though several points of difference are manifest.

Entamœba minuta was so called by Elmassian on account of its small size. According to his description, it does not coincide with our ameba. Hartmann believed this type to be the same as *Entamœba coli.* Kuenen and Swellengrebel, on the other hand, believe it to be identical with *Entamœba tetragena,* with which viewpoint the author concurs.

Entamœba hartmanni, n. sp., is distinguished from other *coli* amebæ by its minute size (4 to 13μ), the structure of the nucleus, and the markedly characteristic, thin, bacteria-like chromidia. This finding of von Prowazek coincides in some points with our own, without, however, being identical.

CONCLUSIONS.

The non-pathogenic tetragenous ameba described here multiplies in the intestine of man, without producing any noticeable symptoms of dysentery.

This non-pathogenic ameba is distinguished morphologically and biologically from *Entamœba tetragena* Viereck and *Entamœba coli* Lösch *emend.* Schaudinn.

The vegetative forms of the non-pathogenic tetragenous ameba may be divided into two groups, large and small. The large vegetative form is found in the normally evacuated or the diarrheal stools

obtained after purgation. The small form is found in the soft stools. This form may be transformed into cysts, or may reproduce itself by binary fission. Cysts are found in relatively large numbers in the soft and formed stools.

Experimentally, the new tetragenous ameba may be readily distinguished from the pathogenic variety. The vegetative forms of the dysentery ameba produced the disease in 91 per cent of cases when introduced *per anum*, and in 50 per cent where the cysts were introduced *per os*. Similar infection experiments undertaken on twenty-three young cats, *i.e.* ten experiments with the vegetative form of the non-pathogenic tetragenous ameba *per anum*, and thirteen experiments with the cysts *per os*, failed to produce pathological conditions. The intestines of the animals macroscopically and histologically failed to show any of the changes of dysentery.

Hence we may conclude that the new type of tetragenous ameba described here is non-pathogenic for man and for young cats.

We desire to express our appreciation to Professor R. Inada and Professor S. Ogawa for the assistance which they have afforded us in our work.

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EXPLANATION OF PLATES.

PLATE 44.

FIGS. 1 to 4. Large vegetative form.

FIG. 1. Mononuclear ameba showing ingested masses.

FIG. 2. Same as Fig. 1, showing bacteria in vacuole.

FIG. 3. Binuclear ameba (simple division).

FIG. 4. Tetranuclear ameba (multiple division).

FIG. 5. Young amebæ after schizogony.

FIG. 6. Nucleus and cytoplasm of large vegetative ameba.

FIGS. 7 to 10. Small vegetative forms.

FIG. 7. Mononuclear ameba with distinct ectoplasm.

FIG. 8. Same as Fig. 7, showing bacteria.

FIG. 9. Binuclear ameba; nucleus with chromatin particles.

FIG. 10. Several chromidia in cytoplasm.

FIGS. 11 to 18. Cyst formation.

FIG. 11. Mononuclear cyst with small central vacuole, showing cyst wall.

FIG. 12. Same as Fig. 11, with large central vacuole, in process of formation. Cyst wall fully developed.

FIG. 13. Same as Fig. 11. Nuclear division. Chromidia surrounding vacuole.

FIG. 14. Nuclear division without large central vacuole.

FIG. 15. Constriction of nucleus.

FIG. 16. Spindle formation of nucleus.

FIG. 17. Binuclear cyst; nuclei still close together. The central vacuole has disappeared.

FIG. 18. Same as Fig. 17, showing division of a nucleus.

PLATE 45.

FIGS. 19 to 42. Cyst formation.

FIGS. 19 to 22. Simple division of nucleus.

FIGS. 19 and 20. Simple division into two.

FIGS. 21 and 22. Cyst with two nuclei, which have arisen after simple division.

FIGS. 23 to 26. Nuclear division of degeneration forms.

FIGS. 23 and 24. Nucleus with four chromatin particles at nuclear membrane.

FIG. 25. Swollen nucleus with four daughter nuclei.

FIG. 26. After the disappearance of the nuclear membrane, four daughter nuclei appear in the cyst.

FIGS. 27 to 30. Four daughter nuclei arising from chromidia in cytoplasm.

FIGS. 27 and 28. Enlarged mother nucleus and chromidia in cytoplasm.

FIGS. 29 and 30. Four daughter nuclei formed from chromidia.

FIG. 31. Binuclear cyst. The nuclear membrane is thin; the nucleus contains several chromatin masses.

FIG. 32. Same as Fig. 31. Mass of chromidia.

FIG. 33. Trinuclear cyst, spindle formation, and division by reduction.

FIG. 34. Same as Fig. 33. Division of karyosome.

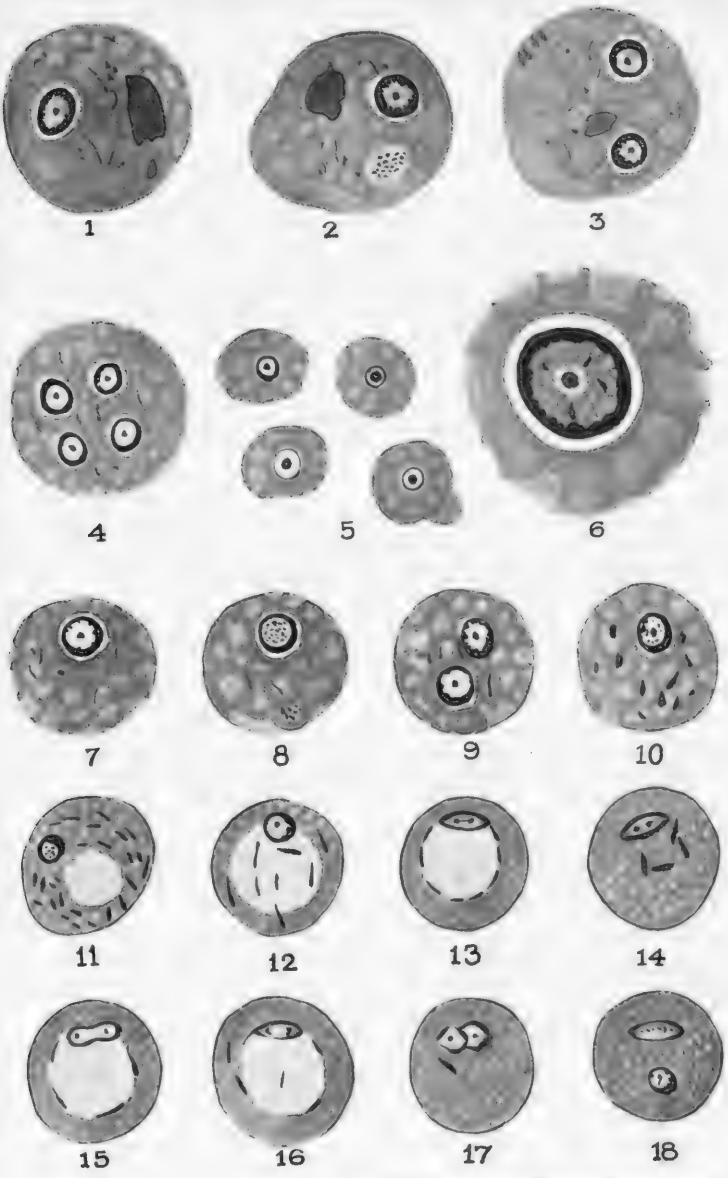
FIG. 35. Binuclear division. Double reduction.

FIG. 36. Nuclear division. Reduction.

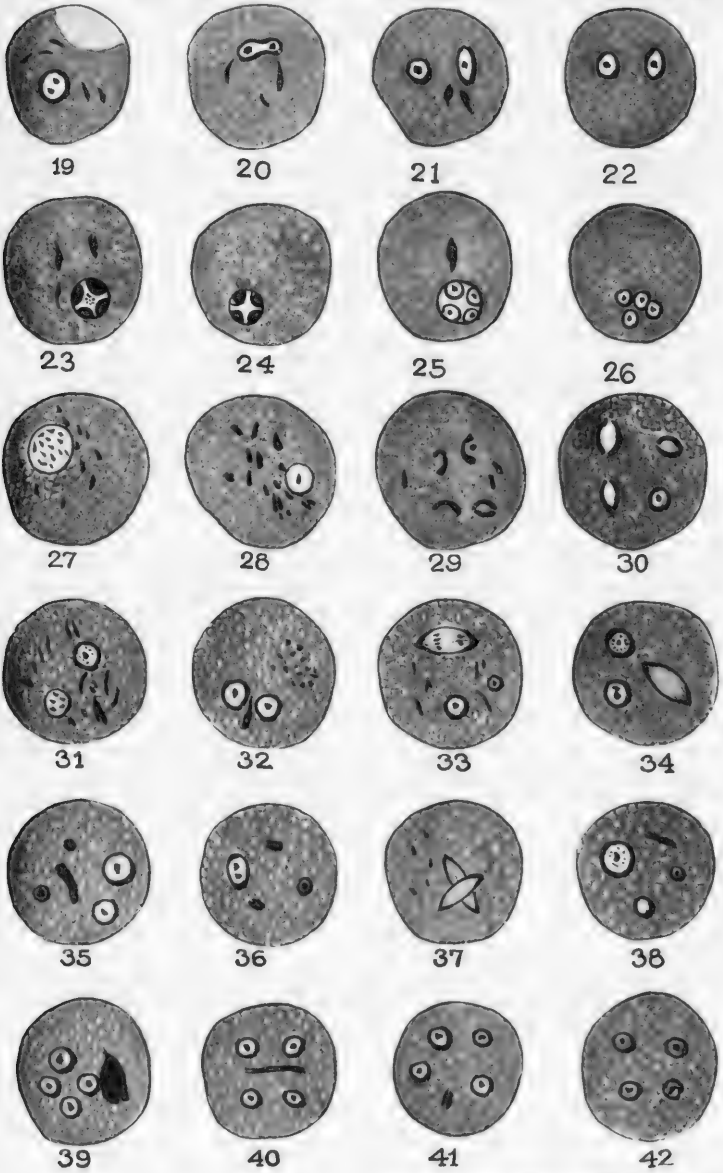
FIG. 37. Spindle formation of two nuclei.

FIG. 38. Trinuclear cyst; one nucleus is very small.

FIGS. 39 to 42. Tetranuclear cyst with and without chromidia.



(Shimura: Non-pathogenic tetragenous ameba. I.)



(Shimura: Non-pathogenic tetragenous ameba. I.)

THE RAT AND POLIOMYELITIS: AN EXPERIMENTAL STUDY.

By HAROLD L. AMOSS, M.D., and PETER HASELBAUER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 1, 1918.)

Under the title of "The rat and infantile paralysis: a theory," Richardson[1] has brought together such hypothetical data in favor of the rat and its parasite, the flea, as the active agents in the transmission of poliomyelitis as he could assemble. He has also brought these evidences to bear on the epidemiology of the disease and has concluded that:

"In the transfer of the infection from the rat to man, the agency of the flea is assumed, although the possible contamination of food by rodent excretions might well be considered. The insect transfer might well be simply mechanical or it might require a preliminary cycle of development of the virus in the flea..... The foregoing theoretical considerations have been put forward as explaining better than any other hypothesis as yet submitted, the epidemiological facts as observed in infantile paralysis."

Richardson further states that extended experimental investigation will be necessary to determine the validity of his theory.

Various kinds of animals have from time to time been incriminated in the spread of epidemic poliomyelitis. Perhaps the one most often cited is poultry because of the paralytic disease, limber-neck, to which they are subject. Doty[2] in his epidemiological report of the New York epidemic of 1916 gives consideration to and excludes from account the rat as an agent of transmission of the disease, and he quotes Professor L. O. Howard to the effect that no marked epizootics among rats have been noted in connection with the larger epidemics of infantile paralysis.

Along with the failure to discover a reservoir of the causative microorganism outside man, by inoculating the nervous tissues from animals suspected to have suffered from poliomyelitis into monkeys,

[1] Richardson, M. W., *Boston Med. and Surg. J.*, 1916, clxxv, 397.

[2] Doty, A. H., Special investigation of poliomyelitis, 1916, Department of Health, City of New York, 40.

unsuccessful attempts have been made to induce paralysis in various kinds of animals, including the white rat, by direct injection of infected nervous tissues into the brain.[3] It is, however, not evident that any systematic effort has been made to detect the virus of poliomyelitis in the rat or to discover whether, when experimentally injected, it is capable of surviving in that animal. Richardson refers in a parenthesis to a statement by Rosenau[4] that while he has apparently been able to produce paralytic disease in rats by inoculation of the virus, yet the experimental data are not sufficiently advanced to justify conclusions. As no confirmation of this important point has been offered in the 2 intervening years, we may assume that convincing evidence has not been obtained.

EXPERIMENTAL.

Our experiments have taken two directions. In the first place, we undertook to transmit poliomyelitis to monkeys by employing for inoculation the central nervous organs of wild rats caught in the localities in Brooklyn in which numerous cases of the epidemic disease prevailed in the summer of 1916. Through the cooperation of the Deputy Commissioner of Health, Dr. John S. Billings, we secured a considerable number of rats. The animals, trapped in cages, were chloroformed and the central nervous and other organs carefully removed and preserved in 50 per cent glycerol in the refrigerator. In the next place, we injected active poliomyelitic virus (suspensions of brain and spinal cord of paralyzed monkeys) into the brain of white rats. As pressure effects which may cause death in a few hours are readily produced, several rats are injected at once. The purpose was to determine whether the virus is capable of surviving in the brain of the rat for any length of time even though poliomyelitis is not induced. Should the virus be quickly destroyed, the conclusion would be justified that the rat organism is not favorable to the virus, from which the further deduction, that this animal improbably serves as a reservoir of the virus in nature, might be made.

[3] Flexner, S., *J. Am. Med. Assn.*, 1910, lv, 1105.

[4] Rosenau, M. J., cited from Richardson,[1] p. 400.

Series I. Inoculation of Organs of Rats into Monkeys.

Experiment 1.—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 1, 2, and 3 collected in Brooklyn. 2 cc. of the suspension were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the nervous organs of the same rats. No symptoms whatever developed.

Experiment 2.—April 11, 1917. 10 per cent suspension was made of the brain and spinal cord of Rats 4, 5, and 6 obtained through Dr. Billings. 2 cc. were injected intracerebrally and 15 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having developed, the injections were repeated with a 10 per cent suspension prepared in the same manner from the nervous organs of the same rats. The animal never showed symptoms.

Experiment 3.—April 11, 1917. The spleen, liver, and kidney of Rats 1, 2, and 3 were made into a 10 per cent suspension. Of this, 2 cc. were injected intracerebrally and 12 cc. intraperitoneally into a *Macacus rhesus*. April 25. No symptoms having appeared, the inoculations were repeated in the same manner and with a 10 per cent suspension prepared from the same viscera. No symptoms developed subsequently.

The significance of this experiment is obvious. Materials from six rats collected in Brooklyn in the vicinity in which the epidemic of poliomyelitis was severe, were injected into three *Macacus rhesus* monkeys under conditions sufficing to incite infection in these animals provided the poliomyelitic virus had been present in the internal organs in any considerable amount and of any real virulence. Moreover, not only did the monkeys fail to respond to a single inoculation of large quantities of the organs of the rats, but they failed equally to react to a second injection of this material made 2 weeks later. It may therefore be concluded that none of the six rats tested carried demonstrable amounts of the poliomyelitic virus.

The next tests are a repetition of those carried out by Amoss[5] on the power of survival of an active virus of poliomyelitis when injected into the brain of rabbits. The tests had been made in the course of a critical examination of the contentions of Rosenow[6] and

[5] Amoss, H. L., *J. Exp. Med.*, 1918, xxvii, 443.

[6] Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *Science*, 1916, xliv, 614; *J. Am. Med. Assn.*, 1917, lxviii, 280.

his coworkers and Nuzum[7] and his associates that poliomyelitis may be induced in rabbits by means of streptococci. Amoss[5] found that the virus of poliomyelitis when injected into the brain of rabbits did not survive there as long as 7 days. The rabbit was therefore regarded as unfavorable to the mere presence within its body of the true virus of poliomyelitis although permitting, on the contrary, streptococci both to survive and multiply in its organs.

Series II. Survival of the Virus in Rats.

Experiment 4.—Under ether anesthesia 0.15 cc. of a suspension of equal parts of active glycerolated monkey virus (spinal cord) and isotonic salt solution was injected intracerebrally into each of four white rats. Two died from pressure within 24 hours. The remaining two were observed for 7 and 14 days respectively and then etherized. Neither developed any symptoms, and on autopsy showed any lesions at the site of inoculation. In each instance the brain tissue surrounding the site of inoculation was made into a suspension with equal parts of isotonic salt solution and injected intracerebrally into a *Macacus rhesus* monkey. No symptoms of poliomyelitis developed.

Experiment 5.—Five white rats were given intracerebral injections of 0.1 cc. of a 50 per cent suspension of active virus in the manner described in the previous experiment. Four succumbed to pressure, and only one survived. The rat which survived and which had shown no symptoms was etherized on the 4th day and the excised site of inoculation in the brain suspended in 2 cc. of isotonic saline solution and injected intracerebrally into a *Macacus rhesus*. The monkey remained well. Because of the brief period of time elapsing between the inoculation of the rat and of the monkey, this experiment was repeated, with the same result.

The tests thus far conducted indicated that the virus of poliomyelitis was incapable of surviving as long as 4 days in the brain of the rat. The question arose whether this result might not be accounted for not by the rapid destruction of the injected virus, but by the small amount which was introduced; in other words, whether the minimal quantities of virus, capable of being injected into the brain without causing pressure effects, were not below the infecting dose when transferred with the rat's brain tissue to the monkey. As a matter of fact 0.1 to 0.15 cc. of the 50 per cent suspension of the spinal cord contains from 10 to 50 minimal lethal doses of the virus for monkeys

[7] Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *ibid.*, 1916, lxvii, 1437; 1917, lxviii, 24.

according to its initial potency. However, the next experiment was designed to answer this question and it showed that the failure to infect is not due to subminimal doses of virus introduced into the rat.

Experiment 6.—A white rat was given an intracerebral injection of 0.1 cc. of a 50 per cent suspension of the virus. $1\frac{1}{2}$ hours later, and after the recovery from ether, the animal was killed with ether and the brain removed. The site of inoculation was excised, suspended in isotonic salt solution, and injected intracerebrally into a *Macacus rhesus*, of which the following is the history:

May 20, 1918. Received intracerebrally 1 cc. of the suspension described. May 26. Somewhat excited. May 27. Tremor, ataxia, weakness of left and complete paralysis of right arm. May 28. Prostrate, etherized.

The autopsy showed typical lesions of poliomyelitis confirmed by microscopic examination of the medulla, spinal cord, and intervertebral ganglia.

DISCUSSION.

The above experiments bring out a remarkable similarity between the tests on the rat and those made by Amoss[5] on the rabbit. Indeed, it now appears that the rat is an even more unfavorable host for the virus than is the rabbit, since in the former animal an effective dose of the virus for the monkey was no longer detectable at the expiration of 4 days. This fact is hardly consistent with the theory that the rat constitutes a natural reservoir in nature of the virus of poliomyelitis. Rather the experiments would indicate that the rat's organism is wholly unadapted to its multiplication and survival.

CONCLUSIONS.

The central nervous organs and other viscera of six rats, collected in a district in Greater New York in which many cases of epidemic poliomyelitis occurred, have been proved incapable of inciting, on inoculation, experimental poliomyelitis in *Macacus rhesus* monkeys.

The virus of poliomyelitis injected into the brain of white rats does not survive there as long as 4 days in a form or in amounts sufficient to cause infection when inoculated intracerebrally into monkeys.

The failure of the virus injected into the brain of rats to incite infection in monkeys is not due to the quantity introduced, since at the

expiration of $1\frac{1}{2}$ hours after the injection, the excised inoculation site when injected into the monkey caused typical experimental poliomyelitis.

It does not appear probable, therefore, that the rat acts in nature as the reservoir of the virus of poliomyelitis.

SPIROCHÆTA HEBDOMADIS, THE CAUSATIVE AGENT OF SEVEN DAY FEVER (NANUKAYAMI).*

FIRST PAPER.

BY YUTAKA IDO, M.D., HIROSHI ITO, M.D., AND HIDETSUNE WANI, M.D.

(From the First Medical Clinic of the Imperial University in Kyushu, Fukuoka, Japan.)

PLATE 46.

(Received for publication, February 20, 1918.)

INTRODUCTION.

There prevails in the province of Fukuoka a disease known as *nanukayami*, or seven day fever, the symptoms of which are like those of atypical Weil's disease. The latter does not usually show any icterus. Seven day fever has a sudden onset with fever, languor, congestion of the conjunctivæ, muscle pain, disorders of digestion, and swelling of the lymphatic glands. Moreover, albuminuria and leukocytosis appear in the earlier stages. Subsequently clouding of the vitreous humor may arise. The disease does not prevail in the cities, *e.g.* Fukuoka, but is restricted to the country. It runs a short course and apparently causes no fatalities. It has been disputed for a long time whether seven day fever and Weil's disease are identical or distinct entities.

Inada examined many cases of seven day fever in 1909 and 1910, and in the latter year he published his views to the effect that the two diseases were independent. As at that time the causative agent of Weil's disease, *Spirochæta icterohæmorrhagiæ*, had not yet been discovered, a final decision was not possible. Ido and Wani observed many cases of seven day fever in the autumn of 1916, and determined through epidemiological and immunological studies that this affection was distinct from Weil's disease.

We have been engaged since 1916 in the study of the etiology of seven day fever, and in October of that year ascertained that the causative agent of seven day fever is also a spirochete which resembles

* Read before the Medical Congress of Japan, April, 1917, and published in *Nippon Naika Gakkai Zasshi*, 1917, v, No. 5.

Spirochæta icterohæmorrhagiæ, but is separable from it. Further study showed that the field mouse (*Microtus montebelli*) is a carrier of this spirochete.

The first of our studies were carried out, as stated, in 1915, and were confined to six human cases of seven day fever. In the autumn of 1917, we were enabled to study twenty-five more cases, and material from twenty-three of the cases was used to inoculate guinea pigs. In twenty instances the results were found to be positive either by detection of the spirochetes or determination of the occurrence of immune reactions. The twenty successful cases had been examined within the first 5 days of the appearance of symptoms of the disease. Moreover, we discovered that the spirochetes in the blood of the patients (Figs. 1 to 3) and also in the urine of a convalescent patient stained by Giemsa's solution. More complete data concerning these cases will be published later.

EXPERIMENTAL.

Isolation and Identification of the Spirochete.

The method of investigation followed was similar to that employed by Inada and Ido in the study of Weil's disease. Six cases have been studied since 1915. The blood and urine of patients were injected intraperitoneally into various animals—guinea pigs, rabbits, mice, and rats.

A guinea pig, inoculated October 1, 1916, with the blood of Patient 1, died on October 27 without having shown conspicuous symptoms. A few spirochetes were found in the kidneys, stained according to Levaditi's method.

In the meantime we observed still another patient (Case 2). The blood from Patient 2 was injected intraperitoneally into three guinea pigs, two rabbits, and three mice. Of these, the three guinea pigs showed fever on the 5th to the 6th day and died on the 7th to 9th day. One of the guinea pigs exhibited a slight icterus and all showed hemorrhage in the lungs. The liver showed many spirochetes on dark-field illumination.

As the form and movement of the spirochetes were similar to those of *Spirochæta icterohæmorrhagiæ*, we at first mistook them for that

organism, and accordingly regarded the condition as Weil's disease. But a more exact observation of the postmortem examinations of the guinea pigs brought out a striking difference in comparison with those of the guinea pig which had died of infection with known *Spirochæta icterohæmorrhagiæ*. Thus, although one of the guinea pigs manifested icterus, it was of very slight degree; and the hemorrhages were much less than in experimental spirochætosis icterohæmorrhagica. Moreover, the lymphatic gland swelling was very prominent. On microscopic examination the lymphatic enlargement was traceable to hyperplasia, while in experimental Weil's disease the swelling is due to hyperemia and hemorrhage. Hence it may be said that the postmortem appearances are more suggestive of seven day fever than of Weil's disease.

We next undertook to determine whether the spirochetes present in the liver of the experimentally infected guinea pig (Fig. 4) were actually *Spirochæta icterohæmorrhagiæ* or another variety. For this purpose we employed convalescent serum of cases of Weil's disease and immune serum of the horse inoculated with *Spirochæta icterohæmorrhagiæ*. These sera contain spirochetolytic and spirocheticidal substances active against this spirochete. A goat was immunized with the new spirochete and its serum tested against *Spirochæta icterohæmorrhagiæ*. We therefore carried out Pfeiffer's tests in two ways. The results in both instances were negative: anti-*icterohæmorrhagiæ* serum was without effect on the new spirochete, and the antiserum from the goat was equally ineffective upon *Spirochæta icterohæmorrhagiæ*.

The next step was to determine whether cross-immunity existed *in vivo*. Guinea pigs which have recovered from experimental spirochete infection are refractory to reinoculation. Hence we injected intraperitoneally the spirochetes of seven day fever into three healthy guinea pigs and let them recover from the infection induced. After the disappearance of the spirochetes from the blood, they were injected with liver emulsion or pure cultures containing many *Spirochæta icterohæmorrhagiæ;* they developed typical experimental Weil's disease. Again we induced in four guinea pigs experimental Weil's disease, and treated them with the specific antiserum. The animals recovered, after which they were injected intraperitoneally with liver

emulsion which contained many of the new spirochetes. All the guinea pigs developed typical experimental disease corresponding to seven day fever.

These experiments indicate that cross-immunity in the guinea pig to the two spirochetes is absent. Hence it would follow that *Spirochæta icterohæmorrhagiæ* and the spirochete obtained from patients with seven day fever are independent organisms, although in both their form and movements they are strikingly alike.

The next point was to establish the fact that the spirochetes present in the guinea pigs actually came from the patients. In this connection we had already learned that this species of spirochete is never present in healthy guinea pigs, while it is not possible to produce the effects described in guinea pigs by the injection of the blood of healthy persons or of patients suffering from other diseases. Hence we conclude that the spirochetes actually existed in the blood of the seven day fever patients and were transferred by injection to the guinea pigs in which the experimental form of the disease was induced.

We carried the proof still further. We studied the effect of serum of convalescents from seven day fever upon this spirochete, for we assumed that it contained a specific immune body. Should this be the case, then the simultaneous injection of spirochetes and the convalescent serum must fail to induce infection in the guinea pig. This proved to be true, as the guinea pigs so treated remained well; while other guinea pigs inoculated with a mixture of the same spirochetes and the *icterohæmorrhagiæ* serum developed the typical disease and died. From these facts we believe that the spirochetes derived from patients with seven day fever are the causative agent of that disease.

But if this spirochete is the causative agent of seven day fever as it occurs in Fukuoka, it must be present in all cases. We tested this point by injecting the blood of patients into guinea pigs; in two cases on the 3rd and 4th days, in three cases on the 6th, and in one case on the 8th day of the disease. We obtained a positive result only in the case tested on the 6th day. At first this was an inexplicable result but it was cleared up later when we came to study the susceptibility of the guinea pig to this spirochete. The young is much more sensitive than the old guinea pig. The latter does not respond either to inoculation

with the hepatic emulsion or with pure culture containing abundant spirochetes; while, on the contrary, young animals easily react typically. Only older animals were used in the first experiments, hence the failures. Unfortunately this point cannot be completely investigated until next autumn as seven day fever prevails only at that time of the year. In the meantime we arrived at the conclusion regarding the spirochete as the cause of seven day fever by indirection by studying the spirochetolytic and spirocheticidal action of the blood serum of the eighteen persons who had once suffered from seven day fever. We made the Pfeiffer test with the serum with our spirochete and for control with *Spirochæta icterohæmorrhagiæ*. Of the eighteen cases, fourteen yielded an immune body which acted upon these spirochetes, while in no instance was a specific immune antibody for *Spirochæta icterohæmorrhagiæ* found. Hence we conclude that the spirochete is the causative agent of seven day fever.

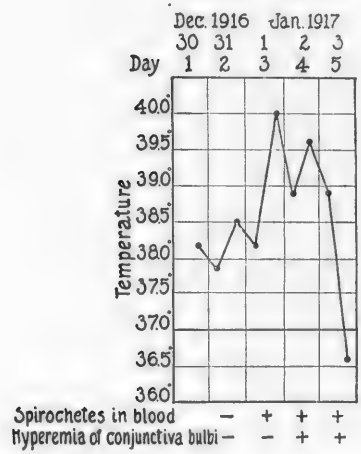
We could now carry our experiments a step further to the detection of the natural carrier of the organism, which proved to be the field mouse (*Microtus montebelli*), just as the rat is the carrier of *Spirochæta icterohæmorrhagiæ*.

According to the tests performed up to the present, 3.3 per cent of the field mice examined show the spirochete in the kidneys (Fig. 5) and in the infected animals they are present in the urine. In movement they resemble *Spirochæta icterohæmorrhagiæ*. Finally, it may be stated that regions in which field mice abound are the regions in which seven day fever occurs. We have called the causative organism *Spirochæta hebdomadis*.[1]

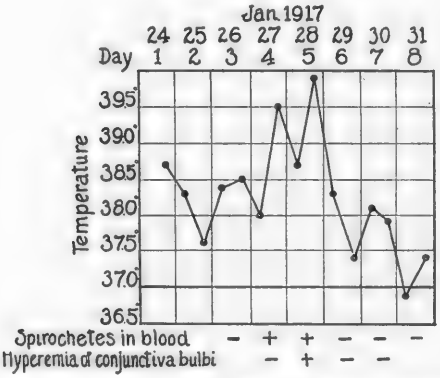
As has been stated, three guinea pigs which developed symptoms following blood inoculation showed spirochetes in the liver by dark-field illumination. The inoculation of urine into guinea pigs was without effect. The results in guinea pigs both with blood and urine were different from those following inoculation from cases of Weil's disease, as fewer infections were obtained with the blood of cases of seven day fever, probably because of the age and size of the guinea pigs employed, and no infections were obtained from the urine.

Transfer from an infected guinea pig to a healthy one sometimes succeeds with the spirochete of seven day fever, but not always.

[1] The author has adopted the corresponding designation of *Spirochæta nanukayami* in the Japanese form of his paper.

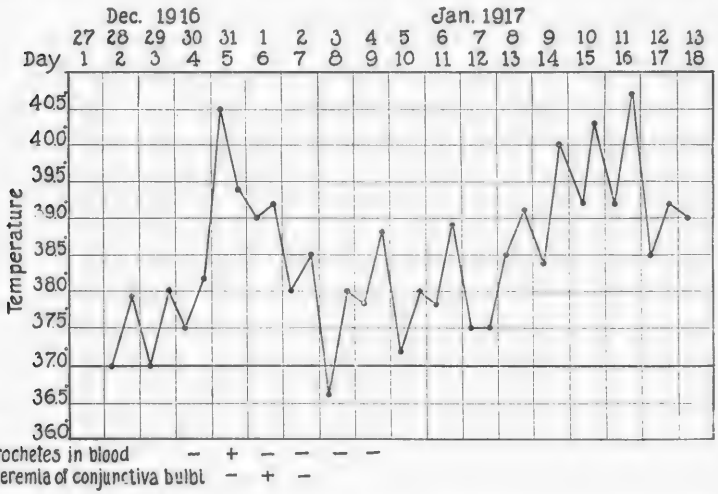


TEXT-FIG. 1. Temperature curve of a guinea pig experimentally infected with seven day fever. Died on the 5th day. No icterus. Postmortem examination positive. Spirochetes abundant in the liver.



TEXT-FIG. 2. Temperature curve of a guinea pig experimentally infected with seven day fever. Died of icterus on the 8th day. Postmortem examination positive. No spirochetes in the liver.

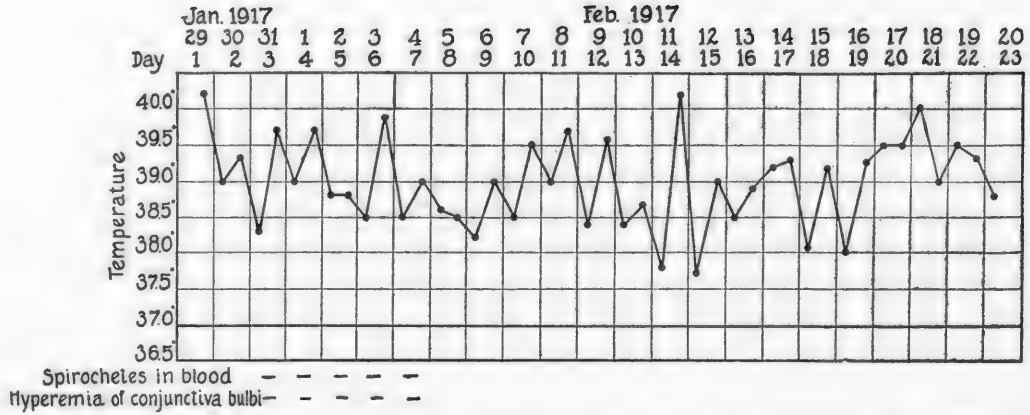
This is another point of difference from *Spirochæta icterohæmorrhagiæ*. Moreover, in certain guinea pigs in which the spirochete of seven day fever appeared in the blood and fever was present, the spirochete afterwards disappeared spontaneously. These animals either recovered entirely or died subsequently although no spirochetes were present (Text-figs. 1 to 4). As has been stated, we came to the conclusion that the larger guinea pigs were insusceptible and hence the failure to infect them. We arranged a set of experiments so as to



TEXT-FIG. 3. Temperature curve of a guinea pig experimentally infected with seven day fever. Died on the 18th day. No icterus. Postmortem examination positive. Spirochetes found in the kidney but not in the liver.

inject a quantity of hepatic emulsion, 1 cc. for every 100 gm. of animal weight. Most of the guinea pigs weighing more than 200 gm. either did not become sick or did not die, while guinea pigs of about 150 gm. weight became infected and died (Table I).

Because of the inconstancy of the infection in guinea pigs, it is difficult or impossible to keep a strain of the spirochete alive by successive transfer to these animals. But this deficiency can be overcome by artificial culture. In order to accomplish transfer from guinea pig to guinea pig the surest way is to inject into the peritoneal cavity



TEXT-FIG. 4. Temperature curve of a guinea pig experimentally infected with seven day fever. Killed on the 23rd day. Postmortem examination: Hemorrhage in the lungs. Spirochetes found in the kidney and urine and immune body in the blood serum.

½ cc. of blood containing the spirochete taken by cardiac puncture. Successful infections can also be accomplished in some instances by skin inoculation with and without previous abrasion, or even *per os*.

The incubation period in guinea pigs fluctuates between 2 and 8 days, being shortest after intraperitoneal injection of cardiac blood and longest after dermal inoculation.

The symptoms in the guinea pig recall those of seven day fever in man. There are anorexia, fever, congestion of the conjunctivæ, emaciation, anemia, hemorrhages, and leukocytosis. But all the

TABLE I.

Intraperitoneal Injection of Spirochetes of Seven Day Fever into Guinea Pigs.

Experiment No.	Body weight.	Amount of hepatic emulsion injected into the peritoneal cavity.	Spirochetes in the blood.	Icterus.	Course	Autopsy.	Spirochetes in the liver.
	gm.	*cc.*					
1	490	4.9	−	−	Normal.		
2	470	4.7	−	−	Died on 9th day.	+	−
3	440	4.4	−	−	Normal.		
4	259	2.6	+	−	"		
5	250	2.5	+	−	Died on 7th day.	+	−
6	232	2.3	+	+	" " 7th "	+	−
7	167	1.6	+	−	" " 6th "	+	+
8	144	1.4	+	−	" " 4th "	+	+

symptoms are slighter than those that appear after inoculation of *Spirochæta icterohæmorrhagiæ*. The temperature is 38–40°C., and continues from 1 to 3 days, when it falls by crisis. The jaundice, when it appears, comes on with the fall in fever. Hemorrhages are slight, as a rule, except in the lungs. Besides being present in the lungs the hemorrhages appear in the abdominal walls, retroperitoneal connective tissues, and the serous membranes; but they are relatively small and few. The external hemorrhages are inconspicuous (Table II).

When spontaneous recovery ensues, the spirochetes first disappear from the blood, then the congestion of the conjunctivæ abates, and

lastly the fever abates. Immune bodies appear in the blood in about 1 week and about that time the spirochetes may be found in the urine.

Besides guinea pigs we inoculated rabbits, mice, and rats with the blood of patients with seven day fever (Table III). With the exception of the rabbits, none of the latter animals showed any symptoms.

Rabbits.—Sixteen rabbits were injected intraperitoneally with 1 cc. of hepatic emulsion per 100 gm. body weight. On the 2nd to the 6th day spirochetes were present in the blood, fever appeared on the 2nd or 3rd day, rarely on the 4th day, and then fell. Young animals of 200 to 500 gm. in weight were more responsive than old animals, and they sometimes succumbed on the 4th or 5th day when

TABLE II.

Percentage of the Mortality, Icterus, and Hemorrhage of Guinea Pigs Experimentally Infected with Seven Day Fever and Spirochætosis Icterohæmorrhagica.

	Spirochætosis icterohæmorrhagica.	Seven day fever.
	per cent	*per cent*
Mortality	100.0	60.8
Icterus	99.0	17.4
Nosebleed	78.0	4.4

spirochetes can be found in the liver. From the 6th day no more could be detected. In older animals the spirochetes have entirely disappeared from the blood on the 4th or 5th day. Young rabbits may show icterus but usually no hemorrhages; old rabbits develop no typical symptoms.

Mice.—Three mice were given ½ cc. of blood or hepatic emulsion intraperitoneally. On the 4th day spirochetes were detected in the blood, but they soon disappeared; no symptoms developed.

Field Mice.—Ten field mice were injected intraperitoneally with ½ to 1 cc. of blood or hepatic emulsion. None showed symptoms, but five showed spirochetes in the urine.

House Rats.—Two house rats only were studied but no results were obtained.

Dermal Infection.—Thirteen guinea pigs were employed for this purpose. The animals are fixed so that they cannot lick off the inoculated material. The hair is clipped from the abdomen and the skin moistened with sterile water. In six animals the skin was scarified so that the abrasions barely reached the corium. In seven guinea

TABLE III.

Animal Experiments with the Blood and Urine of Patients with Seven Day Fever.

Case No.	Age.	Date.	Day of disease.	Experimental animal.	Material for inoculation.	Dose.	Result.	Icterus.	Hemorrhage.	Spirochetes in the liver.	Spirochetes in the kidney.
	yrs.	*1915*				*cc.*					
3	14	Aug. 22	6	Guinea pig (2).	Blood.	2.0	—	—	—	—	—
4	41	Sept. 11	3	Guinea pig (2).	Blood.	2.0	—	—	—	—	—
				Rabbit (1).	"	5.0	—	—	—	—	—
				White rat (1).	"	1.0	—	—	—	—	—
				Mouse (2).	"	0.5	—	—	—	—	—
5	34	*1916* Sept. 11	6	Guinea pig (2).	Blood.	2.0	—	—	—	—	+(1) −(1)
				" "	Urine.	3.0	—	—	—	—	—
				Mouse (2).	Blood.	0.5	—	—	—	—	—
				"	Urine.	0.5	—	—	—	—	—
1	20	Sept. 11	4	Guinea pig (3).	Blood.	2.0	—	—	—	—	—
				Mouse (2).	"	0.2	—	—	—	—	—
2	53	Oct. 16	6	Guinea pig (3).	Blood.	2.0	+	+(1) −(2)	+	+	+
				Rabbit (2).	"	5.0	—	—	—	—	—
				Mouse (3).	"	0.5	—	—	—	—	—
6	19	Nov. 10	8	Guinea pig (3).	Blood.	2.0	—	—	—	—	—

pigs no scarification was made. On each was dropped $\frac{1}{2}$ cc. of hepatic emulsion containing about 10 spirochetes per microscopic field. It was allowed to dry for about half an hour, when the animals were released and returned to their cages. Among the six scarified animals four became infected; among the seven not scarified five developed symptoms. Of the four former three died on the 5th to the 6th day,

and of the five latter all died on the 8th to the 9th day. Pure cultures have also been used for dermal infection.

Oral Infection.—Six guinea pigs were each given by mouth 2 cc. of an hepatic emulsion similar to the one used for dermal inoculation. Of these, five developed symptoms and died on the 6th to the 13th day of the typical disease. We conclude, therefore, that the spirochete can penetrate the intact gastrointestinal mucosa.

Portal of Entry.—The portal of entry of the spirochete into the human body has not yet been minutely studied. The literature of the disease gives certain suggestions; namely, entrance by way of the skin surfaces. In such instances swelling of the regional lymphatic glands was present, and the patients had been workers in the fields or forests, and often suffered skin abrasions. In conformity with this is the observed fact of a case of laboratory infection from the pricking of the finger with the needle of a syringe contaminated with the blood of an infected guinea pig. The attack was typical of seven day fever. We made certain studies on the incubation period of the disease arising in man through dermal infection and concluded that it was about 6 days.

Channels of Excretion.—We also undertook to study whether the spirochetes leave the human body, and if so how. Urine, bile, and feces from inoculated animals were studied by dark-field illumination. In nine out of thirteen guinea pigs the spirochete was detected in the urine: thus in three of six animals dying on the 6th, in two of three killed on the 16th, and in one each of animals killed on the 11th, 18th, 19th, and 23rd day. The urine of nine infected guinea pigs was injected intraperitoneally into nine other guinea pigs of which five acquired the typical disease. Bile, feces, and intestinal contents never conveyed the infection. The conclusion reached is that as with *Spirochæta icterohæmorrhagiæ*, the chief excretory path is by way of the urine. If the spirochetes are present in the bile, feces, or intestinal contents, they are too few to cause infection on inoculation.

CONCLUSIONS.

A new species of spirochete which we have called *Spirochæta hebdomadis* has been described as the specific etiological agent of

seven day fever, a disease prevailing in the autumn in Fukuoka and other parts of Japan.

This spirochete is distinguishable from *Spirochæta icterohæmorrhagiæ* to which it presents certain similarities.

Young guinea pigs are susceptible to inoculation with the blood of patients and to pure cultures of the spirochete, and those developing infection exhibit definite symptoms suggestive of those of seven day fever in man.

The blood serum of convalescents from seven day fever contains specific immune bodies acting spirochetolytically and spirocheticidally against the specific spirochetes, but not against *Spirochæta icterohæmorrhagiæ*.

The field mouse (*Microtus montebelli*) is the normal host of the spirochetes, which have been detected in the kidneys and urine of 3.3 per cent of the animals examined.

The endemic area of prevalence of seven day fever corresponds with the region in which field mice abound.

We wish to express our appreciation to Professor R. Inada for his assistance in this work.

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Ido, Y., Hoki, R., Ito, H., and Wani, H., The field mouse as a carrier of *Spirochæta nanukayami*, the causative agent of seven day fever, *Nippon Naika Gakkai Zasshi*, 1917, v, No. 5.

Ido, Y., Ito, H., and Wani, H., A report on the study of the causative agent of *nanukayami* (second paper), *Tokyo Ijishinshi*, 1917, Nos. 2052 and 2053.

EXPLANATION OF PLATE 46.

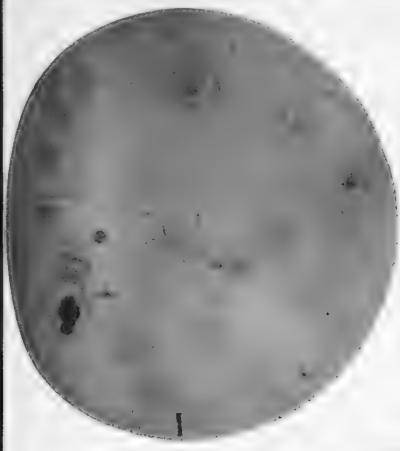
FIG. 1. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 4th day of illness. × 700.

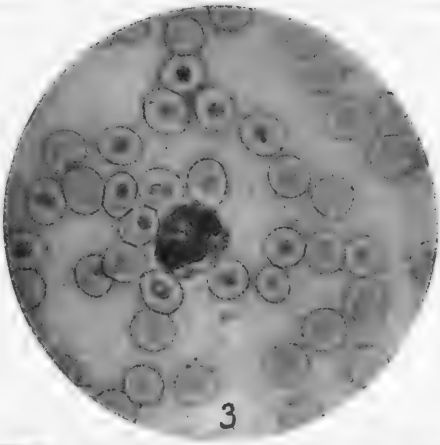
FIG. 2. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 4th day of illness. × 700.

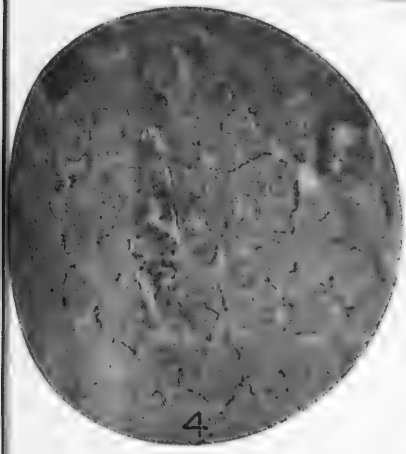
FIG. 3. Microphotograph of *Spirochæta hebdomadis* in human blood. Giemsa stain. 3rd day of illness. × 700.

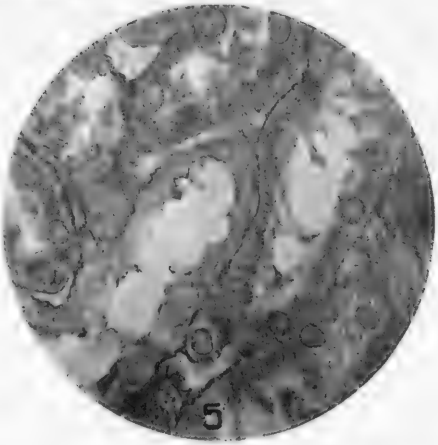
FIG. 4. Spirochetes in the liver of a guinea pig. Silver impregnation.

FIG. 5. Spirochetes in the kidney of a field mouse. Silver impregnation.









(Ido, Ito, and Wani: Seven day fever.)

A REPORT ON ANTIMENINGITIS VACCINATION AND OBSERVATIONS ON AGGLUTININS IN THE BLOOD OF CHRONIC MENINGOCOCCUS CARRIERS.

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Plates 47 and 48.

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Following an outbreak of epidemic meningitis at Camp Funston, Kansas, in October and November, 1917, a series of antimeningitis vaccinations was undertaken on volunteer subjects from the camp. Major E. H. Schorer, Chief of the Laboratory Section at the adjacent Base Hospital at Fort Riley, offered every facility at his command and cooperated in the laboratory work connected with the vaccinations. In the camp, under the direction of the Division Surgeon, Lieutenant Colonel J. L. Shepard, a preliminary series of vaccinations on a relatively small number of volunteers served to determine the appropriate doses and the resultant local and general reactions. Following this series, the vaccine was offered by the Division Surgeon to the camp at large, and given by the regimental surgeons to all who wished to take it.

Preliminary Series.

The preliminary series of vaccinations was carried out in the 342nd Field Artillery Regiment through the courtesy of Colonel Nugent and Major Czar C. Johnson, surgeon of the regiment. This organization volunteered *en masse* in response to the call issued by the Division Surgeon and offered a most promising opportunity for an extended series of observations. Moreover, only one case of meningitis had developed in the 342nd Field Artillery and the regiment had recently been covered in the search for meningococcus carriers. Dur-

ing the first experience the vaccination of known carriers was avoided and this regiment appeared to be free from them.

Choice and Preparation of Vaccine.—That at least two serologically distinct disease-producing types of meningococci exist has been known since Dopter[1] in 1909 described the "parameningococcus" obtained first from the nasopharynx and then from the blood and spinal fluid of active cases of epidemic meningitis. Following Dopter's discovery Wollstein[2] confirmed the serological distinction between normal or regular meningococci and parameningococci by a comparative study of agglutination, opsonization, and complement fixation of the two types, concluding that:

"Because of the variations and irregularities of serum reactions existing among otherwise normal strains of meningococci it does not seem either possible or desirable to separate the parameningococci into a strictly definite class. It appears desirable to consider them as constituting a special strain among meningococci not, however, wholly consistent in itself."

In a more recent study based on absorption of agglutinin and agglutination tests Gordon[3] has distinguished four groups of meningococci which he calls Types I, II, III, and IV. Groups I and II are distinct and correspond to the known types. They are responsible for 75 to 85 per cent of the cases of epidemic meningitis. Other meningococci he separates into Types III and IV. Tulloch[4] finds, however, that definite group relationships exist between Types I and III, and II and IV, so that a I–III group type and a II–IV group type can be distinguished. These and certain other strains can be classified only by the absorption of agglutinin test, since they agglutinate in two or more of the type sera. Type II also 'appears to include a complex subgroup and shows considerable variation among the cocci comprised therein." Nicolle, Debains, and Jouan,[5] employing a rapid method of agglutination or flocculation (without incubation) in low dilu-

[1] Dopter, C., Étude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques), *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

[2] Wollstein, M., Parameningococcus and its antiserum, *J. Exp. Med.*, 1914, xx, 201.

[3] Gordon, M. H., The definition of the meningococcus, *National Health Insurance, Med. Research Committee, Special Rep. Series, No. 3*, London, 1917, 10.

[4] Tulloch, W. J., A study of the mechanism of the agglutination and absorption of agglutinin reaction, together with an examination of the efficacy of these tests for identifying specimens of the meningococcus isolated from 354 cases of cerebrospinal fever, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

[5] Nicolle, M., Debains, E., and Jouan, C., Sur les méningocoques et les sérums antiméningococciques, *Bull. et mém. Soc. méd. hôp. Paris*, 1917, xli, 878; *Ann. Inst. Pasteur*, 1918, xxxii, 150.

tions of immune horse serum, have also reported four types of the meningococcus which they designate A (Gordon I and III, regular meningococcus), B (Gordon II and IV, parameningococcus), C, and D. Types A and B are common, Types C and D relatively rare. They find that certain meningococci are related to two or more of their specific types and must therefore be classed as "indeterminates."

In a recent article Mathers and Herrold[6] readily distinguish the two main types by agglutination, and group around them most of their atypical strains. The strains which fail to agglutinate in type sera have nevertheless similar antigenic properties, since "monovalent serum prepared from these strains agglutinates in a specific way meningococci belonging to one or the other of the large biologic groups."

It appears, therefore, that while two distinct types or groups of meningococci may be clearly differentiated, most of the other organisms that do not fall strictly within these two groups are either intermediates or irregular variants of one or the other. Meningococci of other types in as far as they can be distinguished are only occasionally responsible for a case of epidemic meningitis.

In order to cover the two pathogenic types of meningococci and variants of them, it has become the practice in this country, following the methods of Flexner and Jobling[7] and Amoss and Wollstein,[8] to prepare highly polyvalent therapeutic sera by the repeated injection of a number of representative strains with the object of producing a serum which would protect against any pathogenic strain that might be encountered. For protective inoculation high polyvalency would seem to be equally desirable, but the choice of strains for human vaccination is limited by the fact that only a few injections are practicable, whereas the production of therapeutic serum requires a long series of inoculations in which immunity is gradually built up by the repeated injection of small doses of each antigen. The inclusion of any considerable number of strains in a vaccine for human use might defeat the purpose of the vaccination by the introduction of toxic amounts of bacterial protein without a sufficient quantity of any one specific

[6] Mathers, G., and Herrold, R. D., Observations on meningococcus carriers and on the bacteriology of epidemic meningitis, *J. Infect. Dis.*, 1918, xxii, 523.

[7] Flexner, S., and Jobling, J. W., Serum treatment of epidemic cerebro-spinal meningitis, *J. Exp. Med.*, 1908, x, 141.

[8] Amoss, H. L., and Wollstein, M., A method for the rapid preparation of anti-meningitis serum, *J. Exp. Med.*, 1916, xxiii, 403.

antigen to give rise to protection against its given strain. But since 70 to 85 per cent of the cases of epidemic meningitis are caused by meningococci of the two main types, the limitation of the strains in a vaccine to representatives of these types would seem to be the rational procedure, holding out hope of protection against a large proportion of disease-producing strains.

Therefore, for the vaccine used at Camp Funston only three organisms were chosen from the stock of The Rockefeller Institute, but the vaccine may be regarded as having represented the two main types of the meningococcus. It was also anticipated that the epidemic at Camp Funston might furnish some strain or strains of meningococci not closely related to the main types and which ought therefore to be included in the vaccine, but a type study at Fort Riley showed that the sixteen strains recovered from active cases in December and January would be covered by a vaccine containing normal and para strains.

Method of Preparing Vaccine.—The vaccine used was made in the laboratory of The Rockefeller Institute. 16 hour growths on 1 per cent glucose agar in Blake bottles were washed off with isotonic salt solution, like strains pooled, and the concentrated suspensions immediately heated to 65°C. for 30 minutes to kill the cocci and inactivate the autolytic ferment. Experiments have shown that this temperature does not impair the antigenic properties of the organism and the intact cocci are less toxic than their autolyzed products.

Following the usual tests for purity and sterility the suspensions were standardized, diluted in 0.85 per cent salt solution, mixed in equal proportions of the three strains in concentrations of 1,000 million, 2,000 million, 4,000 million, and 8,000 million cocci per cc. and preserved with 0.35 per cent tricresol.

All the injections in the preliminary series were made strictly subcutaneously with a fine needle (No. 25) at the insertion of the deltoid muscle, usually in the left arm unless a recent vaccina "take" had occurred. Then the right arm was used. The tincture of iodine used as antiseptic was then sponged off with alcohol.

For the determination of dosage and the study of reactions and antibody formation six groups of about 50 men each were chosen from the various companies in the regiment. Successive groups received

increasing doses of vaccine in a series of three injections at 4 to 10 day intervals according to the schedule in Table I.

About 25 men of Groups I, II, III, V, and VI gave preliminary (control) blood samples for the study of immunity reactions on the day of the first injection and a later specimen was obtained from as many vaccinated men as were available on the 8th to 10th day after the third injection.

At Major Johnson's direction First Lieutenant Serge Androp, Medical Corps, obtained reports from the men at inspection the morning following the vaccinations in regard to the resulting local and general reactions.

TABLE I.

Vaccination Schedule, Preliminary Series.

Group No.	1st injection.	Interval.	2nd injection.	Interval.	3rd injection.	Interval. Blood samples taken.
	millions	*days*	*millions*	*days*	*millions*	*days*
I	500	4	1,000	4	2,000	8
II	750	9	1,500	10	3,000	9
III	1,000	9	2,000	10	4,000	9
IV	1,500	8	3,000	8	8,000	10
V	2,000	7	4,000	7	8,000	10
VI	2,000	6	5–6,000	6	10,000	9
VII	2,000	7	4,000	8	8,000	17

Dosage and Reaction.—The determination of the dosage of vaccine for subsequent groups followed from the reports of the reactions produced by the given doses. It was considered important to increase the doses gradually in order to locate closely the zone of mild reactions and to avoid unexpectedly severe results. Accordingly, the vaccinations were begun with the injection of 500 million cocci, and this initial dose was increased in successive groups by 250 or 500 million until it had reached 2,000 million. For the second and third doses in each group, the first dose was usually multiplied by two and by four. Thus it usually happened that a given dose had already been used as a second or third in one group before it was tried as the first dose in a later series.

Only two reactions of any consequence were reported from doses of less than 2,000 million cocci. An officer in Group II who explained

that he had long been hypersensitive to foreign protein and had reacted severely to the typhoid and paratyphoid vaccinations developed a severe local and general reaction, with headache and malaise, after the first injection of 750 million cocci. A second officer in Group II was similarly affected after a second injection of 1,500 million cocci and was confined to bed part of the following day. The two reactions after these small doses were merely transient, but they demonstrate a factor of individual susceptibility which was found to be of considerable importance in the determination of proper doses of vaccine.

When an initial dose of 2,000 million was reached (Group V) general reactions began to appear with greater frequency. None of the men in this group felt ill enough to report at sick call the following morning, but nine men stated upon question that they had felt feverish the preceding night. Four of them had had headache also and one reported a chill. Arms were moderately sore at the site of injection, but not sore enough to interfere with routine duties.

From this time on, a small number of the men in each group reported some local or general discomfort following the vaccination. The symptom most frequently mentioned was a "feverish sensation" often accompanied by headache, which was sometimes severe enough to cause loss of sleep. Morning temperatures, when taken, were reported normal. In a few instances there was transient nausea, malaise, or aching joint pains, and three reactions were initiated with a chill. Eight men had general reactions after the first and second doses, or after the second and third, and three complained of discomfort after all three injections.

On the whole the reactions produced by the vaccine in the preliminary series were mild as compared with those that occasionally follow injections of typhoid or paratyphoid prophylactic and there was little complaint among the men. The occurrence of an occasional reaction of greater severity even with the smaller doses, and increasing local tenderness after the injection of the larger doses of vaccine led to the choice of relatively lower doses for the general series throughout the camp rather than the attempt to push the dosage up to the limit of endurance. Later experience fully justified this decision. Doses of 2,000, 4,000, and 8,000 million had already been decided upon when Group VI was given a second injection of 5,000 or 6,000

million and a third injection of 10,000 to determine the relative security of the chosen doses. The injection of 10,000 million cocci caused general reactions in eleven men, but none of them was confined to bed or relieved from duty following the injection.

Finally, a seventh group of 99 men received the chosen doses of vaccine at weekly intervals about a week before the corresponding injections were given in the camp at large and served as a final check on the dosage. On giving the third injections to Group VII it was noted that many of the men still had a small painless area of subcutaneous induration at the site of the former injections. This persistent induration has been personally experienced after injection of meningococcus vaccine. It disappears gradually, leaving no trace. A more general discussion of reactions is reserved for the report on the larger vaccination series.

Immunity Reactions.—Agglutination is the reaction of choice for the study of antibody production with the meningococcus. It is the most delicate as well as the most specific, and is most easily made on a large number of sera and in multiple dilutions. In studying agglutination, however, several factors have to be taken into account aside from the obvious requirement of a carefully standardized technique. One of the most important is the relative agglutinability of various strains of meningococci and their response to one or both of the type (normal and para) immune bodies. Meningococci vary also in antigenic power and not always in consonance with their agglutinability, so that it is sometimes profitable to use certain type strains as antigens and other strains to test antibody production.

Most of the control sera taken in advance of vaccination from men of Groups I, II, III, V, and VI were used up in agglutination tests against the vaccine strains in a dilution of 1:10. Except in one instance in which the para strain was partially agglutinated, the results of these tests were uniformly negative. When the first of the serum specimens, taken on the 8th and 10th days after vaccination and tested in 1:10 dilution against the vaccine strains, also gave negative results, it was decided to collect all the available sera for later study in lower dilutions and against more easily agglutinable strains. This study was subsequently made at The Rockefeller Institute. A certain number of sera from each group was examined by macroscopic

agglutination with a modification of the Wright method in dilutions of 1:2 and higher against the following strains from the stock of The Rockefeller Institute:

No. 8. A "regular" which is also agglutinated in low dilutions of para serum.
No. 10. An intermediate which agglutinates in both "regular" and para serum.
No. 60. One of Dopter's paras which is relatively easily agglutinable and was represented in the vaccine.

On account of the limited amounts of serum available and the low dilutions required, the following method of agglutination was employed:

Capillary glass tubing of an internal diameter of 2 mm. is drawn out into capsules 8 to 10 cm. long (Fig. 1). For use the ends are snapped off and a nipple is slipped on one end and folded double, giving accurate control of the aspirated fluids. A file mark measures equal volumes of serum and salt solution for successive dilutions, which are made in the capsule and deposited in a row on a plate of solid paraffin in a Petri dish. The paraffin plate is conveniently cleaned by melting off a thin layer in hot water. Equal amounts of a serum dilution and a meningococcus suspension are measured in the capsule, mixed on the paraffin plate, and drawn up to form a column about 1 to 1.5 cm. long. Four or five such specimens, separated by air bubbles, are sealed in a capsule for incubation. Only one dilution of a serum should be used in a single capsule, but several meningococcus suspensions (4 billion per cc.) may be tested against it, as the admixture of specimens in the capsule is inappreciable. The capsules are woven through holes in a card which designates their contents, and incubated in a horizontal position for 24 hours at 55°C. Complete agglutination is indicated by a widespread flocculated sediment of organisms (Fig. 2). The flocculi are distributed through the clear fluid on rolling the tube briskly between the palms (Fig. 3). Absence of agglutination corresponds to a smooth line of sediment, which goes into even suspension on whirling. Partial agglutination is easily recognized in a combination of the two types of sediment. Results are read with the unaided eye or under a small hand lens.

As stated above, most of the control sera had been exhausted before this more comprehensive series of tests was undertaken. There remained fourteen normal control sera from Group VI, and it happened that the practically negative results obtained with the sera of Groups I, II, and III admit these specimens as controls, since the small doses of vaccine used in these groups did not give rise to demonstrable antibody formation. To these may be added twelve sera from supposed non-contacts obtained in New York City.

TABLE II.

Agglutination Tests with Sera of Vaccinated Men.
Dilution 1:2.

Controls, not vaccinated.	Strains.			Group I.	Strains.			Group II.	Strains.		
	8	10	60		8	10	60		8	10	60
N 1	−	−	−	5	++	++	++	71	−	−	−
N 2	−	−	−	8	−	−	−	73	−	−	−
N 3	−	−	−	11	−	−	−	77	++	++	−
N 4	−	−	+	13	−	−	−	79	−	−	−
N 5	−	−	−	14	−	+	−	81	−	−	−
N 6	−	−	−	15	−	+	−	88	−	−	−
N 7	−	−	−	20	−	−	−	94	−	−	−
N 8	−	−	−	21	+	−	−	100	−	−	−
N 9	−	−	−	24	−	++	−	112	−	−	−
N 10	−	−	−	25	−	−	−	113	−	−	−
N 11	+	−	−					114	−	+	−
N 12	−	−	−					115	−	−	−
Group III.				Group IV.				Group V.			
122	−	−	−	173	+	+	+	227	−	++	+
125	−	−	−	179	−	+	−	233	++	++	+
126	−	−	−	180	−	+	−	234	++	++	−
127	−	−	−	183	−	+	−	237	+	++	+
134	−	−	−	185	−	++	−	239	+	+	+
135	−	−	−	186	−	−	−	240	++	++	−
140	−	−	−	188	−	−	−	246	−	+	−
142	−	−	−	192	−	+	−	250	−	++	+
147	−	−	−	195	−	+	−	251	−	++	++
152	−	−	−	197	−	−	−	252	++	++	++
155	−	−	−	202	−	++	−	253	−	++	+
160	−	−	−	206	−	+	−	254	−	++	+
				210	−	+	−	255	−	++	+
				212	−	++	−	256	++	++	+
				215	−	+	−	257	−	++	++
								259	−	+	−
								261	++	++	+
								262	+	++	++
								263	++	++	++
								264	++	++	++
								267	−	++	++
								268	−	++	++

++ indicates complete agglutination, + partial agglutination, − no agglutination.

TABLE II—*Concluded.*

Group VI.	Before vaccination (controls). Strains.			After vaccination. Strains.			Group VII.	Strains.		
	8	10	60	8	10	60		8	10	60
269				−	−	−	322	−	++	++
270				−	+	+	323	−	++	++
271	−	+	+	−	+	−	324	−	+	++
273	+	+	−	−	++	++	326	−	++	++
274				+	++	−	331	−	+	+
278	−	−	−	+	++	−	334	−	++	++
284	−	−	−	−	+	−	335	−	++	++
286	−	−	−	+	++	+	354	−	++	++
287	−	−	−	−	++	−	380	−	++	++
288	−	−	−	−	+	−	395	−	+	+
289	−	−	−	−	+	−	397	−	++	++
290	+	+	−	+	++	+	399	−	++	+
291	−	−	−	+	+	−	406	−	++	+
292	−	−	−	−	++	−	409	−	++	++
294	−	−	−	−	+	−	415	+	++	++
296	−	−	−	+	++	++				
299				−	+	+				
310				−	+	+				
317				−	+	−				

Table II shows the absence of agglutination in Groups I, II, and III with few exceptions. In three cases only was more than a partial agglutination of one strain in the 1:2 dilution observed. The titers in these cases ran as follows:

Group.	Serum.	Strain.		
		8	10	60
I	5	1:32	1:8	1:8
	24	0	1:32	0
II	77	1:8	1:32	0

It may be assumed, therefore, that the number of unvaccinated men whose sera would agglutinate any of these chosen strains of meningococci is small, and on the basis of this assumption the observation that twelve of fifteen sera from Group IV agglutinate Strain

10 partially or completely is unmistakable evidence of antibody formation through the agency of the vaccine. Group V, in which the dosage reached that chosen for the camp at large, confirms this evidence of reaction, for all the twenty-two sera studied contained antibodies for the meningococcus. In some instances the more easily agglutinable strains were agglutinated in a dilution of 1:32 or 1:64 (Table III).

TABLE III.

Agglutinin Titers of Sera from Group V.

Serum.	Strains.		
	8	10	60
227	0	1:32	1:32
233	1:2	1:32	1:8
234	1:2	1:4	0
237	1:2	1:16	1:4
239	1:2	1:16	1:2
240	1:2	1:64	0
246	0	1:2	0
250	0	1:128	1:8
251	0	1:32	1:32
252	1:32	1:64	1:8
253	0	1:32	1:8
254	0	1:64	1:8
255	0	1:32	1:2
256	1:2	1:64	1:2
257	0	1:64	1:16
259	0	1:2	0
261	1:2	1:32	1:2
262	1:2	1:32	1:32
263	1:4	1:32	1:32
264	1:2	1:64	1:64
267	0	1:8	1:8
268	0	1:64	1:8

The final evidence of antibody production is furnished by a direct comparison of sera from Group VI taken before the first vaccination with sera taken 9 days after the third injection of vaccine, in which the appearance or increase in agglutinins is observable in all but two instances. The presence of agglutinins in the sera from Group VII merely confirms the previous findings. *It may, therefore, be stated*

that the injection of well tolerated doses of meningococcus vaccine is followed by specific antibody formation in the human body.

The preliminary series of vaccinations, therefore, served to establish the method of injection, the proper dosage for extended vaccination, the reactions which might be expected to follow the chosen doses, and the production of immune bodies in the serum of vaccinated men. On the basis of these findings the vaccine was offered to the camp at large.

General Series.

The vaccine used in the general series of inoculations in the camp was made by Lieutenant Peter K. Olitsky at The Rockefeller Institute by the methods already described. It was shipped to Fort Riley in bulk and was diluted in isotonic salt solution to standard concentration, preservedwith 0.3 per cent cresol and distributed in 50 cc. bottles to the regimental surgeons under Major Schorer's direction. The use of two suspensions, one of 4,000 million cocci per cc. (Vaccine A and B), and the other of 8,000 million (Vaccine C), adjusted the injection volumes to 0.5 and 1 cc. of Vaccine A and B and then 1 cc. of Vaccine C, volumes similar to those for the typhoid prophylactic, with doses of 2,000 million, 4,000 million, and 8,000 million cocci to be given at weekly intervals.

At the direction of the Division Surgeon, the Division Training Officer, Captain Albert Bower, to whom especial thanks is due for his mediation between the regimental surgeons and the laboratory, called a meeting of the surgeons at which the method of giving the vaccine and the results to be expected and observed were fully described and discussed. The surgeons were thus informed of the procedure and object of the vaccination and their cooperation was enlisted, without which little could have been accomplished. The response of the men when the vaccine was offered to them was due in large measure to the interest and example of the regimental surgeons.

According to the statistics of the division headquarters, the total strength of the 89th Division at this time was approximately 25,000 officers and men. Of these, 4,792 (19 per cent) took the first injection, 4,257 (17 per cent) the second also, and 3,702 (15 per cent) completed the series.

Part of the men received the full dosage as planned. About half of those vaccinated, whose third injection was due after February 4, 1918, were given a final injection of 4,000 million, on account of the occurrence of several fairly severe reactions from the larger dose among medical officers at Fort Riley. In some regiments the vaccinations had been completed before February 5.

Reactions.—After the first injections had been given, the opinion was almost universal in the camp that the vaccine caused less general and local reaction than the typhoid prophylactic. In very few regiments was a man excused from duty the following day on account of the reaction from the vaccination. The general feeling was that the second dose caused less reaction than the first, but there were a few men in almost every organization who had reactions of moderate severity, sometimes being confined to bed for the day with headache, joint pains, and nausea. Several cases of looseness of the bowels or transient diarrhea were noted. This symptom had not been encountered before. Careful inquiry in individual cases often elicited the information that men who complained of the effects of vaccination were suffering from mild coryza, bronchitis, etc., at the time of injection.

Among the units who took the third injections before the dosage was reduced, and so received a third dose of 8,000 million meningococci, there were several instances of fairly severe reactions, general and local, which necessitated relief from duty the following day. The reactions were not more severe than those that occasionally follow paratyphoid prophylactic and no untoward results were reported. The large majority of the men seem to have suffered no appreciable reaction whatever. The smaller doses of 4,000 million cocci caused even fewer reactions.

As in the preliminary series, the factor of individual susceptibility was prominent, a few officers and men suffering severely from doses which caused no general discomfort in the great majority of the men. In general, the more severe reactions occurred among the commissioned officers, and especially among the medical officers at the Base Hospital and in the Medical Officers' Training Camp at Fort Riley, due in part perhaps to more confining occupations, higher nervous tension, and more introspection than was common among the enlisted

men. In one regiment, through a misunderstanding, four men were given an initial dose of 8,000 million cocci, which was well tolerated. The surgeon reported:

"One had chill for 30 minutes, headache for 1 hour, slight local reaction.
One had slight headache, slight local reaction.
One had severe local reaction and headache for 24 hours.
One had slight local reaction, headache for 12 hours."

A survey of the reports of the regimental surgeons and of the observations in the preliminary series shows that headache was the most frequent symptom following injection, and accompanied most of the other symptoms encountered. Sometimes the reaction was initiated by a chill or chilly sensation, and a number of men complained of fever or feverish sensations during the following night. Next in frequency came nausea (occasionally vomiting), dizziness, and general "aches and pains" in the joints and muscles, which in a few instances were especially localized in the neck or lumbar region, causing stiff neck or stiff back. A few injections were followed by diarrhea. The reactions, therefore, occasionally simulated the onset of epidemic meningitis and several vaccinated men were sent as suspects to the Base Hospital for diagnosis.

Such transient reactions are illustrated by the following brief protocols:

Individual 1.—C. D., Private, Battery D, 342nd Field Artillery.

Jan. 15, 1918. 1st injection 2,000 million. "Sore arm."

Jan. 22. 2nd injection 4,000 million. "Sore arm, headache."

Jan. 29. 3rd injection 8,000 million. "Began to feel badly about 15 minutes later and had a chill. 3 hours later was sent to the Base Hospital complaining of headache, lumbar pain, stiff neck, and fever, 103°F. No nausea. Went to sleep about 2 hours later and slept well. Had entirely recovered the next morning."

Individual 2.—L. N., Private, Field Hospital Company 354, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. "Slight local soreness."

Jan. 28. 2nd injection 4,000 million. "Slight local soreness."

Feb. 4. 3rd injection 8,000 million. "About an hour and a half after injection, was taken with a severe chill and a subnormal temperature. About 20 minutes later his temperature rose to 103.6°F. and then fell within the next 2 hours to 101°F. Temperature normal the following morning. He complained of no other symptoms."

The most severe illness immediately following vaccination is described by the officer himself as follows:

Individual 3.—J. M. K., First Lieutenant, 314th Sanitary Train.

Jan. 21, 1918. 1st injection 2,000 million. 4.30 p.m. Soreness at site of injection in evening. Slept well.

Jan. 22. Awakened feeling drowsy and listless. Local soreness worse. Pains in back of neck, calves of legs, thighs, lumbar region, and both arms. No appetite. Symptoms grew worse after holding sick call at the Infirmary. Perspired on slight exertion. Felt chilly and warm by turn. Went to quarters and lay in bunk. Soon felt feverish. Developed diarrhea about 11 a.m. Eight movements in 5 hours. Cup of hot chocolate at 1 p.m. Felt nauseated. Vomited six times in next 3 hours. Took sodium bicarbonate, 2 gm. in glass of warm water, promptly vomited. Retained 15 minutes later, and then began to feel better. Felt well next morning but had no appetite. Bowels still loose. Returned to duty. Appetite returned the evening of Jan. 24. Local soreness persisted 3 days.

		Temperature. °F.	Pulse.
Jan. 22,	9.45 a. m.	98.4	86
	1.15 p. m.	99.8	82
	3.45 p. m.	101.0	92
	7.30 p. m.	101.8	108
	10.00 p. m.	100.0	98
Jan. 23,	7.25 a. m.	97.8	98

Jan. 28. 2nd injection 4,000 million. Local reaction severe. Duration 3 days. General reaction, malaise of 24 hours duration.

Feb. 4. 3rd injection 8,000 million. Local reaction severe. Duration 4 days. General reaction, nausea, looseness of bowels (not diarrhea), chilliness, but no fever. Muscle soreness severe and general. Appetite absent for 2 days.

The occurrence of such reactions as those described above was rare but they illustrate the importance of the factor of individual susceptibility. The great majority of the men vaccinated were reported to have had no general symptoms, and very few men were confined to quarters after any dose of the vaccine.

Occurrence of Meningitis after Vaccination.—The records of cases of epidemic meningitis in Camp Funston have been followed up to June 4, 1918, thus covering the period that the 89th Division remained in camp. In the interval between January 21 when the vaccination was started in the camp and June 4, 46 cases of meningitis are reported to have entered the Base Hospital at Fort Riley. Of these patients, three had received one, two, or three injections of antimeningitis vaccine. The following data have been collected in regard to these cases.

Case 1.—L. T., Second Lieutenant, 314th Engineers.

Jan. 21, 1918. 1st injection 2,000 million. Next day neck was rather stiff, slight headache. Well for next 2 days, but working very hard on double duty.

Jan. 25. Fainted at Officers' Call. Felt "under the weather" with indefinite symptoms the next 2 days but remained on duty.

Jan. 28. 2nd injection 4,000 million. This made him feel so much better that he remarked to his friends that he "was getting to be a dope fiend in the stuff and couldn't get along without it."

Jan. 29. Felt worse again, headache, "no ambition." Following days he remained on duty, but tired easily and often felt chilly and feverish, had stiff neck and joint pains.

Feb. 2. Remained in bed. Throbbing headache, became nauseated and vomited twice. Sent to Base Hospital. Lumbar puncture on arrival showed a turbid fluid with many leukocytes and moderate numbers of intracellular Gram-negative diplococci.

Lieutenant T. had a very mild case of meningitis which yielded promptly to serum treatment.

Case 2.—E. H., Corporal, Company C, 354th Infantry.

Jan. 21, 1918. 1st injection 2,000 million. "Never felt anything. Arm not very sore."

Jan. 26. Went on 5 days leave, returning Jan. 31, and so missed second injection.

Feb. 1. "Felt sore all over." Had chills. Frontal headache.

Feb. 2. Headache continued, stiff joints and aching pains. Sent to Base Hospital. Diagnosis, epidemic meningitis.

Corporal H. had a severe case of meningitis but recovered.

Case 3.—J. C. N., Private, Company B, 340th Machine Gun Battalion.

Jan. 24, 1918. 1st injection 2,000 million.

Jan. 31. 2nd injection 4,000 million.

Feb. 8. 3rd injection 4,000 million. Reactions not reported.

Mar. 31. Acute otitis media and right frontal sinusitis.

Apr. 16. Epidemic cerebrospinal meningitis.

Apr. 24. Cerebrospinal fluid found positive for meningococcus for the last time.

Apr. 27. Ulcer of right cornea followed by slight opacity with slight impairment of vision.

May 5. Right facial paralysis which has since improved considerably.

Private N. will be ready for assignment to domestic military service about July 1, 1918.

The examples of Lieutenant T and Corporal H are instructive, since they may well be, and probably are, instances of meningococcus inoculation in individuals in the incubation period of a meningococcus meningitis. Assuming this to be the fact, the indication is that no

harm was done by the procedure. But whether this is the precise fact or not, the cases have no real bearing on the value of antimeningitis vaccination, because the interval between the first inoculation and

TABLE IV.

Agglutination of Meningococci in Sera of Vaccinated Men.
Dilution 1: 2.

314th Engineers.	After 3rd injection.	Strains.			355 Infantry.	After 3rd injection.	Strains.			314th Sanitary Train.	After 3rd injection.	Strains.		
		8	10	60			8	10	60			8	10	60
	days					*days*					*days*			
501	17	−	+	−	535	15	−	−	−	585	17	−	++	+
502	17	−	+	−	536	15	−	++	+	586	17	−	+	+
503	17	−	+	+	537	15	+	++	++	587	17	−	−	−
504	17	−	−	−	538	15	−	++	+	590	17	+	−	−
506*	10	−	++	++	539	15	−	+	−	591	17	−	−	−
509*	10	−	−	−	541	15	−	++	+	593	17	−	+	+
510*	10	−	−	−	544	15	−	+	−	595	17	−	++	++
511*	10	−	++	++	545	15	−	++	+	598	17	+	++	++
512*	10	−	++	+	547	15	+	++	++	600	17	−	+	+
513*	10	−	++	−	549	15	+	+	+	606	17	++	++	++
514*	10	−	+	+	553	15	++	++	++	609	17	−	++	++
515*	10	−	+	−	558	15	+	++	+	610	17	++	++	++
516*	10	−	+	++	560	15	−	−	−	611	17	+	++	+
517*	10	−	−	−	562	15	+	+	−	612	17	−	++	++
518*	10	−	−	−	565	15	−	+	−	613	17	++	++	++
520*	10	+	++	++	566	15	−	−	−	614	17	−	++	++
528	18	−	−	−	568	15	−	−	−	615	17	−	−	−
531*	10	−	+	+	569	15	−	++	+	618	17	+	++	++
532	18	−	+	−	570	15	−	−	−	619	17	−	++	++
533	18	−	−	−	571	15	−	+	−	622	17	−	+	+
534	18	−	−	−	574	15	−	++	+					
					576	15	−	++	++					
					578	15	+	++	+					
					581	15	−	++	+					
					583	15	−	+	+					

* 3rd dose 4,000 million cocci.

the diagnosis of the meningitis was too brief to permit immunization to be effected.

It is clear that Private N developed meningitis at a period at which protection following vaccination should have been present. In his case the vaccination must be considered as having failed to afford

protection. A study of the type of the infecting meningococcus would conceivably have thrown light on the incident, or the incident may merely indicate that occasional individuals fail to be adequately immunized, just as in the analogous procedure of antityphoid inoculation.

Immunity Reactions, General Series.—In addition to the blood samples taken for study in the preliminary series of vaccinations, similar samples were obtained from a group of men in each of three regiments who had completed the vaccination series. The serum specimens were taken at an interval of from 10 to 18 days after the third injections had been given, and were examined for agglutinins by the method already described. The results of these tests are shown in Table IV. The sera of 50 men showed some content of agglutinins; the sera of 16 others failed to do so.

Examination for Agglutinins of the Blood Serum of Chronic Carriers.

While every case of epidemic meningitis is presumed to develop from the carrier state, many observers have noted the rarity of the disease among chronic carriers. The presence of the meningococcus in the nasopharynx is but one of the factors in the accident which results in the bacterial invasion of the body, and the relatively low infectivity of the meningococcus is to be credited to causes of resistance on the part of the carrier host among which may well be the appearance of immune bodies in the blood as the result of the multiplication of meningococci in the nasopharynx.

If the presence of agglutinins for the meningococcus could be demonstrated in the blood of chronic carriers, light would thereby be thrown on the mechanism of this resistance. With the object of detecting the possible existence of meningococcus agglutinins in the blood serum of carriers, a number of such sera have been studied in low dilutions by the capsule method described in an earlier section of this report.

Through the courtesy of Passed Assistant Surgeon J. L. Waterman, United States Naval Hospital, Brooklyn Navy Yard, and Dr. F. S. Westmoreland, Riverside Hospital, New York, opportunity was given to obtain blood samples from a number of chronic carriers of the meningococcus, and the corresponding carrier strains were kindly furnished

by Dr. A. W. Williams of the Department of Health, of the City of New York, or obtained directly from the men themselves. The men studied were known to have harbored meningococci for from 4 to 16 weeks.

20 carrier strains of the meningococcus have been subjected to the agglutination tests in low dilutions with the sera of the hosts from whom they were obtained and the carrier sera were tested against the stock strains of spinal origin that had been studied in the sera of vaccinated men from Camp Funston. 18 of the carrier strains were also

TABLE V.

Control Agglutination Tests with Normal Sera against Carrier Strains. Dilution 1: 2

Normal control serum.	Carrier Strains.																	
	124	125	127	128	129	133	134	136	139	141	147	150	151	153	156	160	161	162
1	−	−	−	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	+	−	++	++	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
5	+	−	+	−	−	−	−	−	−	++	+	−	−	−	−	−	−	−
6	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+	−	+	+
7	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−
8	−	−	−	−	−	−	−	−	−	−	−	−	*	−	−	+	−	−
9	++	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
10	+	+	+	+	+	+	−	−	−	++	++	−	−	−	++	−	+	+
11	+	−	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*No test.

tested with 12 normal sera from supposed non-contacts as controls. The results of these tests are recorded in Tables V to IX. The trials with non-contact sera (Table V) show that with two exceptions the carrier strains are not usually agglutinated in normal sera in a 1: 2 dilution. One of the exceptional strains was partially or completely agglutinated by 9 and the other by 8 of the 12 sera. The other cultures were only occasionally agglutinated in this low dilution of normal serum. One supposedly normal serum agglutinated 11 of the 18 carrier strains. In the presence of sera from their hosts, however,

all but 4 of these carrier strains were agglutinated (Table VI) and the sera in most instances showed agglutinins for stock spinal strains of the meningococcus. Subsequently the sera were reexamined in multiple dilutions, and at this time 2 of those previously found negative agglutinated their strains up to 1:4 and 1:8 (Table VII). The titers of the other sera ran from 1:4 to 1:64.

TABLE VI.

Agglutination of Homologous Nasal Strains and Stock Spinal Strains in Sera of Carriers.

Serum of.	Homologous nasal strains.	Spinal Strains.		
		8	10	60
Carrier 2	++	++	++	++
" 3	−	−	−	−
" 6	+	+	++	++
" 9	++	++	++	+
" 11	+	++	++	−
" 13	++	++	++	++
" 16	++	++	++	++
" 18	+	++	++	+
" 25	+	++	++	+
" 27	++	++	++	++
" 28	++	++	++	++
" 29	++	++	++	++
" 30	+	+	++	+
" 36	+	−	−	−
" 39	++	++	+	−
" 40	+	+	+	−
" 41	−	+	−	−
" 43	−	+	−	−
" 48	++	++	++	−
" 50	−	−	−	−

Appearance of Agglutinins in Sera after Storage.—On numerous occasions we have observed an increase in agglutinating power in sera which have stood at ice box temperature for some time. The fact was reported in a recent paper[9] as having occurred with typhoid agglutinins in guinea pig sera, and Tulloch[1] records an instance in

[9] Gates, F.L., Antibody production after partial adrenalectomy in guinea pigs, *J. Exp. Med.*, 1918, xxvii, 725.

TABLE VII.

Agglutination of Carrier Strains in Homologous Sera.

Serum of.	Homologous Strain.	Agglutination titers.					
		1:2	1:4	1:8	1:16	1:32	1:64
Carrier 2	124	++	+	+	−	−	−
" 3	125	+	+	+	−	−	−
" 6	127	+	++	+	++	−	−
" 9	128	++	++	++	++	++	+
" 11	129	+	++	++	++	++	++
" 13	133	++	+	−	−	−	−
" 16	134	++	++	++	+	−	−
" 18	136	++	++	+	+	−	−
" 27	139	++	++	+	+	−	−
" 28	141	++	++	+	+	−	−
" 36	147	++	+	+	+	++	++
" 39	150	++	++	++	++	+	+
" 40	151	−	+	+	+	−	+
" 41	153	++	*	+	−	−	−
" 43	156	−	−	−	−	−	−
" 46	160	++	++	++	++	++	++
" 48	161	++	++	++	−	−	−
" 50	162	++	+	−	−	−	−

* No specimen.

TABLE VIII.

Appearance of Agglutinins in Carrier Sera after Storage.
Dilution 1: 2.

Serum drawn May 24, 1918.	Examination of May 29.				Examination of June 12.				Examination of June 24.			
	Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.			Homologous Strain.	Spinal Strains.		
		8	10	60		8	10	60		8	10	60
16	−	−	−	−	+	++	++	+	++	No test.		
36	−	−	−	−	+	−	−	−	++	−	+	+
39	−	−	−	−	++	++	+	−	++	−	++	++
40	−	−	−	−	+	+	+	−	−*	−	−*	−
41	−	−	−	−	−	+	−	−	++	−	++	++
43	−	−	−	−	−	+	−	−	−	−	++	++
46	−	−	−	−	No test.				++	−	−	++
50	−	−	−	−	−	−	−	−	++	−	+	+

* + in higher dilutions.

rabbit serum during immunization to the meningococcus. This he explains as due to the presence of constituents of the serum or of the organisms which do not take part in the mechanism of agglutination, but which may be present "in such quantity or in such a physical state that they protect the united antibody-antigen complex from the flocculating action of the electrolytes."

Table VIII shows the appearance of agglutinins in some of the sera under discussion after they had stood, sealed, at 4°C. for some time. The other sera were not examined so early after bleeding. The

TABLE IX.

Agglutination of Stock Strains in Sera of Carriers.

Serum of.	Agglutination titers of homologous strains in rabbit serum.		Agglutination titers of stock spinal strains in carrier sera.		
	Normal.	Para.	No. 8	No. 10	No. 60
Carrier 27	1:400+	1:400	1:2	1 16	1:16
" 28	1:400+	1:100	0	1:32	1:4
" 36	1:400+	1:50	0	1:64	1:4
" 39	1:800	1:100	0	1:32	1:64
" 40	1:400+	1:50	0	1:64	0
" 41	1:400+	1:100	0	1:32	1:8
" 43	1:50	1:100	0	1:32	1:8
" 46	1:800	1:200	0	0	1:16
" 48	1:50	1:100	0	1:32	1:8
" 50	1:400+	0	0	1:8	1:2

inconstancy of the phenomenon has already been described[9] and is implied by Tulloch in his reference to "this particular serum."

A variation of a different order was encountered when ten of the sera studied on May 29 or June 12 were examined on June 24 in multiple dilutions with the stock spinal strains (Table IX). Six of the ten had agglutinated Strain 8 in the 1: 2 dilution. Now all but one serum failed to do so. Strains 10 and 60, however, were agglutinated in various serum dilutions by practically all the specimens examined, including No. 43, which had failed to agglutinate its homologous strain. Agglutinins for these spinal strains are only rarely found in normal sera (Table II).

DISCUSSION.

Heretofore meningococcus vaccines have not been extensively employed for prophylactic immunization, and only a few references are to be found in the literature that relate vaccination experiences. Davis,[10] in 1907, describing animal experiments and the therapeutic use of an autogenous vaccine reported a personal experience in which he suffered a very severe reaction following the subcutaneous injection of a 24 hour slant culture of a meningococcus heated to 65°C. for 30 minutes. Shortly after inoculation, nausea and vomiting were followed by a severe chill, lasting half an hour, and then intense headache, muscular pain, purging, and vomiting of bile. His temperature rose to 103°F., and during the remainder of the day, and in the night following, nausea and vomiting continued, with headache, thirst, and marked prostration. Later symptoms included a diffuse rash, herpes, and in the urine granular, hyaline, and epithelial casts. The reaction subsided gradually. The leukocytes rose to 44,050 on the 3rd day, and the opsonic index reached 2.3 on the 2nd day, returning to normal by the 5th day.

Sophian and Black[11] studied agglutination and complement fixation in serum specimens from ten students who had been vaccinated with two or three doses of a monovalent vaccine. The doses given were 500 or 1,000 million, 1,000 or 2,000 million, and 2,000 million cocci, at 7 day intervals. Their vaccine had been heated to 50°C. for 1 hour. Following the vaccinations they noted malaise, frontal headache, and slight fever with occasionally more severe symptoms; intense frontal or vertical headache, general bodily pain, nausea, vomiting, and a rise of temperature to 102–104°F. Labial herpes was seen. Using a readily agglutinable organism, they found the agglutinin titers of the sera of their vaccinated subjects to range from 1 : 200 to 1: 1,500. Complement was fixed in serum dilutions up to 1: 250.[12] Complement-fixing antibodies were found in low dilutions of the serum of seven of these men after an interval of 2 years.

Sophian and Black refer to Hall's experience in Kansas City in the vaccination of about 280 persons in families in which meningitis had occurred. A number of doctors and nurses were likewise inoculated, and in no instance did the disease occur subsequent to vaccination. About 100 persons in Dallas were vaccinated, but most of them did not complete the vaccination series. Two nurses developed epidemic meningitis some weeks after a series of two inoculations; both recovered.

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[10] Davis, D. J., Studies in meningococcus infections, *J. Infect. Dis.*, 1907, iv, 558.

[11] Sophian, A., and Black, J., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*, 1912, lix, 527. Black, J. H., Prophylactic vaccination against epidemic meningitis, *J. Am. Med. Assn.*, 1914, lxiii, 2126.

[12] Such figures must be accepted with reserve. They are far higher than laboratory experience would lead one to expect after such prophylactic doses of a meningococcus.

Recently Whitmore, Fennel, and Petersen[13] have reported an experience with a polyvalent lipovaccine in which total doses of 40,000 million or 80,000 million cocci were given subcutaneously in one or two injections. 55 men in all were vaccinated. 40,000 million cocci in one dose did not cause any general reaction. Two doses of 40,000 million cocci each at a 3 day interval were followed by two instances of constitutional reaction among 25 men. 5 men received 80,000 million cocci in a single injection which was followed after 24 hours by a moderate general reaction. In the first days after vaccination with the larger doses agglutinin formation was observed against three of the vaccine strains, especially those that respond to antibodies of both the normal and the para type.

These reports from the literature coincide with the present experience with meningococcus vaccine in their descriptions of the reactions that may be expected, and of the appearance of specific antibodies in the blood after vaccination. As Sophian and Black pointed out, the general symptoms indicate some degree of meningeal irritation and occasionally they may simulate the onset of meningitis. The symptoms are not progressive, however, and even though severe, they clear up in a few hours. In one instance in which a lumbar puncture was done on a suspect 3 days after a second dose of vaccine the spinal fluid was found normal. The illness described by Davis is instructive to show the severity of the symptoms which may follow an injection of meningococcus substance many times the proper dose. Whitmore, Fennel, and Petersen, by protecting their vaccine in oil, were able to give much larger doses in a single injection with only moderate constitutional effects.

Whatever may be the relation of agglutinins to specific protection against invasion the agglutination test is recognized as the most reliable indication of antibody formation due to the meningococcus, and is used generally in the standardization of therapeutic sera as an index of potency. With equal reason the presence of agglutinins may be taken as an index of active immunization after vaccination. We do not know the ratio of agglutinin formation to protective power, and can only discover by wide experience what agglutinin titers correspond to relatively complete immunity to meningococcus invasion. It is perhaps significant, however, that the agglutinin titers

[13] Whitmore, E. R., Fennel, E. A., and Petersen, W. F., An experimental investigation of lipovaccines: a preliminary note, *J. Am. Med. Assn.*, 1918, lxx, 427.

of the sera of vaccinated men are of the same order of serum dilutions, namely 1 : 4 to 1 : 64+, as those of chronic carriers of the meningococcus, who are usually refractory to the strains carried. A study of the blood of cases of meningitis which have recovered without serum treatment might be instructive in this connection.

Meningococcus agglutinins appear not to have been found previously in the blood of chronic carriers. Cathoire,[14] in a brief communication, reported that the agglutinin study of carrier sera led to no positive result, but with the Wright technique he was able to show a constant increase in opsonic power compared with sera from normal persons, and he therefore concluded that the relative immunity of carriers is to be explained by a specific change in the serum.

Herrick[15] thinks that the occurrence of relapses in some cases of epidemic meningitis "lends discouragement to vaccine prophylaxis and other measures for the production of immunity." It should be pointed out that relapses are probably caused by reinfection from small pockets in the meninges in which the meningococcus has been walled off, and so permitted to survive therapeutic measures otherwise effective. It may be mentioned in passing that the occurrence of relapses in typhoid fever has been no contraindication to the employment of typhoid prophylaxis. The object of prophylactic vaccination is to oppose the meningococcus at the threshold, and if a systemic invasion precedes the spinal infection, as recent observations tend to show, the building up of antibodies in the blood stream is the means by which a hematogenous incursion is to be combated.

SUMMARY.

1. A meningococcus vaccine suspended in salt solution has been given subcutaneously as a prophylactic to about 3,700 volunteers in three injections of 2,000 million, 4,000 million, and 4,000 or 8,000 million cocci at weekly intervals.

2. These doses rarely caused more than the mildest local and general reactions. Exceptionally a more severe reaction emphasized the

[14] Cathoire, E., Recherche du pouvoir opsonisant du sérum des porteurs sains de méningocoques, *Compt. rend. Soc. biol.*, 1910, lxix, 240.

[15] Herrick, W. W., The intravenous serum treatment of epidemic cerebrospinal meningitis, *Arch. Int. Med.*, 1918, xxi, 541.

presence of an unusual individual susceptibility to the vaccine. In such instances the symptoms were in part those of meningeal irritation and sometimes simulated the onset of meningitis.

3. Specific meningococcus agglutinins have been demonstrated in the blood serum of vaccinated men as compared with normal controls.

4. Moreover, agglutinins have been demonstrated in the blood serum of chronic carriers of the meningococcus. Evidence is thus brought forward that the relative immunity of chronic carriers to epidemic meningitis may be due to the presence of specific antibodies in the blood stream.

EXPLANATION OF PLATES.

PLATE 47.

FIG. 1. Capsule of glass tubing of 2 mm. internal diameter showing points at which it is snapped off for use, and file mark for measuring equal volumes of serum and meningococcus suspension.

FIG. 2. Appearance of agglutination specimens after incubation. (*a*) Complete agglutination. (*b*) No agglutination. (*c*) Partial agglutination.

PLATE 48.

FIG. 3. Appearance of completely agglutinated specimens after shaking.

FIG. 1.

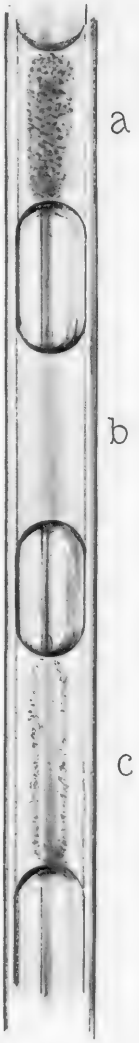

FIG. 2.

(Gates: Antimeningitis vaccination.)

FIG. 3.

(Gates: Antimeningitis vaccination.)

FURTHER INVESTIGATIONS ON THE ORIGIN OF TUMORS IN MICE.

V. The Tumor Rate in Hybrid Strains.

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In former investigations we have tested the cancer rate and cancer age in hybrid strains.[1] In some crosses the cancer rate in both parents was similar; in these cases the offspring followed the cancer rate and age of the parents. In other cases there was a marked difference in the cancer rate and cancer age of both parents. In such crosses we found in the majority of cases either an intermediate result or dominance of the parent with the higher tumor rate. Our results are therefore incompatible with the assumption that has been expressed that in crosses the parent with the lower cancer rate dominates. We found it desirable to continue our observations, especially with a view of increasing the number of crosses in which the cancer rate and cancer age of both parents showed a marked difference. For this purpose we had at our disposal a number of suitable strains or substrains whose cancer rate and age had been carefully followed through a number of years.

We made the following crosses:

1. Silver + English (Offspring of 177a); Strain a.

The two English females died without tumors 7 and 12 months old.

Without tumors.	With tumors.
$F_1 - F_3$ 8 (5 I 1 II 2 III)	14 (6 I 8 II)
36 % (63% I 12% II 25% III)	64 (43% I 57% II)

[1] Lathrop, A. E. C., and Loeb, Leo, *J. Exp. Med.*, 1915, xxii, 713.

2. Silver + English (Sable F_5); Strain c.

The two Sable females that entered into the cross with Silver died without tumor in the first and third age period respectively.

	Without tumors.	With tumors.
F_1	5 (3 I 1 II 1 III)	2 (1 II 1 III)
F_2	4 (3 II 1 III)	7 (3 I 4 II)
$F_1 + F_2$	9 (3 I 4 II 2 III)	9 (4 I 5 II)
	50% (33% I 45% II 22% III)	50% (44% I 56% II)

The combination of Silver and English is of particular interest in view of the great difference in tumor rate between these two strains. The rate of the English varies between 55 and 80% and that of Silver between 7 and 10%. The tumors appear early in both kinds. There attaches an additional interest to this combination because of the fact that the Silver represented originally a substrain of the English. In both crosses a similar result was obtained. The higher tumor rate of the mothers (English) prevailed over the low tumor rate of the (Silver) father. The tumors belong like those of the parents to the first age class. It is improbable that in this case in both hybrid strains an accidental selection of Silver individuals with a higher tumor rate took place, considering the uniformity in the low tumor rate of the Silver throughout several generations.

It is, furthermore, of interest to note that all the female English which entered into these strains died without tumors; but belonging to a strain rich in tumors they nevertheless carried within them the potentiality of a high tumor rate. The realization of this potentiality depends on more or less accidental (external) circumstances. While the English were prolific, but delicate, the Silver were very poor breeders. Both kinds of hybrids were prolific and usually vigorous. In this case, then, high tumor rate of the mother strain was linked with a considerable prolificity; both these characteristics were dominant over the opposite characters of the father strain.

London + Silver.

One London male was mated to three Silver females. Later the same London male was given to Cream females. Four generations were observed. The records of the generations agreed well with each other.

	Without tumors.	With tumors.
Total:	154 (34 I 89 II 31 III)	22 (4 I 11 II 7 III)
	88% (22% I 58% II 20% III)	12% (18% I 50% II 32% III)

The records of the parent strains are as follows:

London (old):		II age class.
	73% (41% I 29% II 30% III)	27% (27% I 43% II 30% III)
London (new):		I age class.
	72% (31% I 40% II 29% III)	28% (40% I 51% II 9% III)
Silver (old):		I age class.
	93% (41% I 45% II 49% III)	7% (50% I 50% II)
Silver (new):		
	90% (33% I 56% II 11% III)	10% (100%II)

In the cross the tumor rate is somewhat better than that of Silver. However, the rate is nearer that of the Silver (mother) than of the London (father). The tumors appear somewhat later than in either parent.

We may conclude that the result is intermediate, but perhaps somewhat nearer the mother strain which in this case has a lower rate. It is a valuable confirmation of our conclusion that the high tumor rate of the cross Silver + English was not due to an accidental selection of Silver mice with higher tumor rate. Mated with the London strain the Silver tumors remain true to their low tumor rate.

If we split off from larger strains definite families which preserve the characteristics of the larger strains and later hybridize the families, the tumor rate and tumor age of the offspring remain in accord with the tumor rate and age of the main strain. Such hybridizations may serve as controls to cases in which the tumor rates of both parents differ. We cite two examples:

A son of Tumor Mouse 344 (English Sable) was mated to a daughter of 328 (English Sable). This same son of 344 was later used for hybridization with German, European + English, 8½, Black Cream, and Cream. Mated to a sister this son of 344 had offspring with a very high tumor rate.

344 + 328

Without tumors.	With tumors.
F_1 F_2 F_4 1 (1 I)	6 (6 I).
14½% (100% I)	85½% (100% I)

(*344* F_1 + *328* F_1) F_2 + *437* F_2 (*437* = *English Sable*)

Without tumors.	With tumors.
$F_1 - F_5$ 6 (3 I 3 II)	21 (15 I 6 II)
22% (50% I 50% II)	78% (71% I 29% II)

In both groups the typical English tumor rate and tumor age were obtained.

8½ + 328

Four daughters of 328 (English Sable) were crossed with an 8½ F_4 male.

Without tumors.	With tumors
F_4 62 (45 I 14 II 3 III)	84 (53 I 29 II 2 III)
42½% (72% I 23% II 5% III)	57½% (63% I 34.6% II 2.4% III)

The tumors belong to the first age class. 8½ had a low tumor rate of 17 %; the tumors belonged to the II age class. In the cross the higher tumor rate and the earlier tumor class of the mother prevailed. These records agree with the records of a similar cross published in our preceding paper.[1] Here the tumor rate was 61 % and the tumors belonged likewise to the first age class. We

see then that in different crosses between 8½ and English Sable the higher tumor rate and the earlier tumor age of the mother strain prevailed.

It was of interest to attempt the reciprocal cross, in which the father was English (344) and the mother belonged to the 8½ strain. Two crosses were made. First 344 (English Sable) was mated to an 8½ F_4 female and after 8½ F_4 had died, 344 was crossed with a young 8½ F_6 female.

English (344) + 8½ F_4.

Without tumors.	With tumors.
$F_1 - F_4$ 83 (31 I 25 II 27 III)	34 (11 I 16 II 7 III)
71% (37% I 30% II 33% III)	29% (32% I 47% II 21% III)

The tumor rate is intermediate, but approaches more closely the low rate of 8½ than the high rate of English. The tumor age is also intermediate between that of 8½ (II age class) and English (I age class).

English Sable (344) + 8½ F_6.

Without tumors.	With tumors.
$F_1 - F_3$ 33 (9 I 12 II 12 III)	5 (3 I 1 II 1 III)
87% (27% I 36½% II 36½% III)	13% (60% I 20% II 20% III)

In this the 8½ tumor rate dominates still more completely than in the former cross. The tumors appear early. The tumor age is apparently influenced by the English father. However, the number of tumor mice is so small in this cross that little value can be attached to the exact determination of the age class.

We see then that while in a cross of 8½ male with English Sable female, the English female dominates, in the reciprocal cross the 8½ female dominates either completely or almost so. As far as the tumor age is concerned, the English influence is still noticeable, but not entirely dominant. We must conclude that the English 344 father was less able to determine the tumor rate and tumor age of the offspring than various English females.

English Sable (344 F_1) + German.

The same 344 male which had been used in the former records was crossed with two rather old German F_4 females.

Without tumors.	With tumors.
$F_2 - F_6$ 16 (5 I 11 II)	29 (17 I 11 II 1 III)
35% (31% I 69% II)	64½% (58½% I 38% II 3½% III)

The tumors stand between the I and II age class, but are much nearer the I age class. The tumor rate of German varies between 50 and 31%. The tumor age varies between the II and I class. In this cross the difference between the two parents is, therefore, not very marked; on the whole the offspring seem to follow more closely the tumor rate of the father. 344 + German were very good breeders and a fairly strong, healthy strain. In former crosses between

English Tan and German, the tumor rates were 49 and 43 %. The tumors belonged to the first age class. We see, therefore, that the crosses between two high tumor strains again show a high tumor rate.

English Sable 344 + (European + English, Daughter of Tumor Mouse 146) = 344 + 146.

The female parent strain had formerly a tumor rate of 28 % (intermediate generations), but in the third generation the tumor rate was as low as 9 %. The tumors belonged to the II age class. The more recent records, which are, however, based on a smaller number of mice, show a tumor rate of 42 %, which is similar to the tumor rate in the first generations. The tumors appear late. The figures for the cross 344 + 146 are as follows:

Without tumors.	With tumors.
$F_1 - F_2$ 20 (3 I 9 II 8 III)	1 (1 II)
95% (15% I 45% II 40% III)	5% (100% II)

The tumor rate is very low. Evidently the low rate of the mother strain dominates. In this case the mother belonged probably to a substrain with a tumor rate lower than that of the average of the strain. The only tumor which was observed in this cross appeared in the II age period. In this cross the effect of the European strain dominates, therefore, over the English strain.

English Sable + (English Silver + I Daughter of No. 10 Nov. 3 Strain).

In this case a high tumor strain (male) (55 to 80 % I age class) was crossed with a medium tumor strain (female) (36 % II age class). The offspring follows the high tumor rate of the father. The old and new records combined give the following figures:

Without tumors.	With tumors
8 (6 I 2 II)	15 (6 I 9 II)
35% (75% I 25% II)	65% (40% I 60% II)

The tumors belong to the first age class. In this cross two high tumor strains enter into combination with a low tumor strain.

English Sable + European Hybrid F_3—(175).

European Hybrid F_3 is (European + No. 10 Nov. 3) F_1. European Hybrids had a tumor rate of 72 % and belonged to the II age class. There were, therefore, two high tumor strains combined in this case.

Without tumors.	With tumors.
$F_1 - F_4$ 10 (7 I 2 II 1 III)	31 (22 I 8 II 1 III)
24½% (70% I 20% II 10% III)	75½% (71% I 26% II 3% III)

The high tumor rate of both parents and the early age class of the father strain prevailed.

A second cross of the same kind in which, however, different individuals were used, was produced.

English Sable (482) + Daughter of 392 (May 1911 Group of European Hybrids).

The daughter of 392 later developed a tumor:

	Without tumors.	With tumors.
$F_1 - F_6$	46 (27 I 15 II 4 III)	38 (17 I 18 II 3 III)
	55% (58% I 33% II 9% III)	45% (42% I 50% II 8% III)

While the tumor rate in the offspring is high, it is lower than in the preceding cross which was of the same kind. The tumors belong here to the II age class of the mother strain. The fourth generation of the cross had in this case a low tumor rate. While certain variations in the tumor rate and tumor age in crosses of the same strains, but into which different individuals enter, do therefore, occur, these variations were in this case not very considerable.

Summary.—(1) Crosses between two substrains which have the same tumor rate and tumor age as the original strain from which they were split off, give the same tumor rate and age as those of the original strain.

(2) Crosses between different strains both of high tumor rate, have high tumor rate; in our case the tumor ages differed and the earlier tumor age of the English strain was dominant.

(3) Two crosses between Silver and English Sable which were both substrains of the English family, but differed markedly from each other in their tumor rate, had the high tumor rate of the mother.

(4) In crosses between English and $8\frac{1}{2}$, the English tumor rate and age prevailed, if the English was used as mother strain, while in two reciprocal crosses the tumor rate was the low rate of the $8\frac{1}{2}$ strain or it approached it. In the tumor age some influence of the English strain was apparently noticeable, although the tumor age was also influenced by the $8\frac{1}{2}$. Whether in this case the individual character of the English father (344) was responsible for the result, we cannot definitely decide at the present time.

(5) In a cross of the same English male (344) with a (European + English), the low tumor rate of the mother prevailed, while in two

crosses in which other English males were mated to females of high and medium tumor strains the English age prevailed and the tumor rate was high.

Crosses between English and Cream.

Crosses between English and Cream are of particular interest in view of the great difference in the tumor rate and tumor age in these two strains. We had previously reported on the following two crosses of this kind.[1]

English Tan (121) + 3 Cream:	42% II age class.
" Sable male (No. 4,444) + Cream:	No tumors.

In one cross an intermediate result was obtained, in the other the Cream strain dominated. It seemed desirable to add to these experiments a number of further crosses between English and Cream.

English Sable (344) + Black Cream.

The 344 substrain of English Sable was the offspring of a son and a daughter of Tumor Mouse 344 (English Sable). This substrain had a very high tumor rate. One of the males of this substrain was mated to a Black Cream female, the tumor rate of which varied between 2 and 10 %, and the tumor age between the II and IV age class.

Without tumors.	With tumors.
$F_1 - F_4$ 71 (36 I 18 II 17 III)	21 (8 I 9 II 4 III)
77% (51% I 25% II 24% III)	23% (39% I 41½% II 19½% III)

The tumor age stands between the I and II age class, but is nearer the II age class. This cross was, therefore, in tumor rate and tumor age intermediate between English and Cream, but somewhat nearer Cream.

(a) (344 + Black Cream) F_1 + Cream (White).

A male of the first generation of the former strain was mated to a Cream female (of white color).

Without tumors.	With tumors.
$F_1 F_3 F_1$ 11 (3 I 2 II 6 III)	0
100% (27% I 18% II 55% III)	0%

This cross was three-fourths Cream and one-fourth English. The Cream prevailed. Tumors did not develop.

(b) English (344) + Cream.

Two males of the 344 English Sable family which differed from those used in the previous cross were mated to Cream females, not identical with the ones which entered into the previous cross.

Without tumors.	With tumors.
130 (61 I 43 II 26 III)	13 (6 I 5 II 2 III)
91% (47% I 33% II 20% III)	9% (47% I 38% II 15% III)
Almost I age class.	

The tumor rate is very low, almost corresponding to that of Cream, but the tumors appear earlier and in this respect perhaps the influence of the English father is noticeable.

English Sable (4,444) + Cream.

A part of this record was reported previously.

Two English Sable males were mated to nine Cream females which had been kept without breeding until they were 9 months old. Most of the daughters of the English father with English Sable females had an offspring with a high tumor rate. A daughter of this English Sable male and English female was mated to ($8\frac{1}{2}$) and the cross showed a high tumor rate.

We have, therefore, no reason to assume that this male did not possess potentiality to a high tumor rate.

These hybrids of English Sable (4,444) + Cream had formerly no tumors.

Without tumors.	With tumors.
$F_2 - F_5$ 170 (44 I 54 II 72 III)	5 (2 I 2 II 1 III)
97.2% (26% I 32% II 42% III)	2.8% (40% I 40% II 20% III)

The Cream again prevailed in this cross. The few tumors which were found appeared relatively early.

Black Cream + English White.

One Black Cream male was mated to ten English females, including 344 F_4, 437 F_4, English Sable F_5.

Without tumors.	With tumors.
$F_1 - F_3$ 86 (33 I 23 II 30 III)	57 (19 I 29 II 9 III)
61% (38% I 27% II 35% III)	39% ($33\frac{1}{3}$% I $50\frac{2}{3}$% II 16% III)

The tumor rate is intermediate between English and Cream and the tumor age stands between the I and II age class. In this cross the influence of the English strain is noticeable to a higher degree than in the majority of the other crosses between English and Cream.

Cream + English (Oct. 1913).

A Black Cream male was mated to a group of English females of the Oct. 1913 generations.

Without tumors.	With tumors.
$F_1 - F_4$ 84 (29 I 27 II 28 III)	25 (6 I 10 II 9 III)
77% (35% I 32% II 33% III)	23% (24% I 40% II 36% III)

The cross is intermediate between English and Cream, but somewhat nearer Cream. The tumor age stands between the II and III age classes. In the age class also the influence of the Cream is marked.

White Cream + White English.

An albino Cream male, son of a Black Cream, was mated to a White English female, the offspring of Tumor Mouse 437 and another tumor mouse belonging to the same group as 437.

Without tumors.	With tumors.
$F_1 - F_3$ 19 (8 I 7 II 4 III)	8 (5 I 2 II 1 III)
71% (42% I 37% II 21% III)	29% (62½ I 25% II 12½% III)

The tumor rate is intermediate between English and Cream. The tumors appeared as early as in the English.

Cream + English (Descendants of English Sable F_1 1,031).

Without tumors.	With tumors.
$F_1 - F_3$ 18 (6 I 7 II 5 III)	8 (1 I 5 II 2 III)
69% (33⅓% I 38⅔% II 28% III)	31% (12½% I 62½% II 25% III)

The tumor rate is intermediate, but the tumor age is that of the Cream; it stands between the III and IV age classes.

One substrain of Cream was Cream Y. Its record was as follows:

Without tumors.	With tumors.
$F_1 - F_4$ 14 (6 I 5 II 3 III)	Tumors were not observed in this substrain.

English Sable + Cream Y.

One English Sable F_4 male was mated to three Cream Y F_1 females.

Without tumors.	With tumors.
32 (14 I 14 II 4 III)	36 (11 I 22 II 3 III)
47% (43¾% I 43¾% II 12½% III)	53% (30⅔% I 61⅓% II 8% III)

The tumor rate is a little lower than that of the English. The tumor age stands between the I and II age classes, but is nearer the II age class, as far as the first and second age periods are concerned. In this instance, then, the result is intermediate between English and Cream in tumor rate as well as in tumor age, but with great preponderance of the English father.

693 + Cream.

693 was 121 (English Tan) + Cream. This male was mated to four Cream sisters which died without tumors. The tumor rate of the offspring of 693 was 42%; the tumors belonged to the II age class.

Without tumors.	With tumors.
$F_1 F_2$ 5 (4 I 1 II)	2 (2 I)
71½% (80% I 20% II)	28½% (100% I)

The number of mice in this strain is small, but the results indicate that the tumor rate will be intermediate between that of the father and mother strains. The tumor rate is certainly higher and the tumors appear earlier than in the Cream.

693 (= 121 (English Tan) + Cream) + 773 (= 8½ + 328 English Sable).

Previous to the mating with the Cream females, 693, which was (121 + Cream F_3) F_1, had been mated to one 8½ + 428 F_1 female.

Without tumors.	With tumors.
14 (10 I 4 II)	21 (14 I 7 II)
40% (71½% I 28½% II)	60% (66⅔% I 33⅓% II)

Tumor rate and tumor age of this strain are that of the English. The father strain had a tumor rate of 42% and belonged to the II age class; the mother strain had a tumor rate of 57½% and belonged to the I age class. The English component dominated in the mother strain. The mother strain and with it the English tumor rate and age class dominated in the complex hybrids.

We may arrange these hybrids in two classes; in the first one the father of the hybrids was English and the mother Cream, while in the second class, the reverse conditions prevailed.

Father: English, Mother: Cream.

English Tan (121) + 3 Cream. 42% II age class. I (nearer English).
" Sable (4,444) + " 0–2.8% C (somewhat earlier tumors).
" " (344) + Black Cream. 236 between I and II age classes. I
(*a*) 344 + Black Cream F_1 + Cream White. C.
(*b*) 344 + Cream. 9% (almost I age class). C (almost I age class).
English Sable + Cream Y. 53 II age class. I (nearly English).
Composite Strains:
693 + Cream (= English Tan + Cream) + Cream. I.
693 + 773 (= 8½ + 328). 60% English prevails through mother strain. E.

Father: Cream, Mother: English.

Black Cream + English White	39% I–II age class.	I (English preponderant).
Cream + English (Oct. 1913)	23% II–III " "	I (Cream ").
White Cream + White English	29% I age class.	I (English tumor age).
Cream + English (1,031)	31% III–IV age class.	I (Cream " ").

I = Intermediate; E = English; C = Cream.

In four strains the father was Cream and the mother English. These four crosses were all intermediate, the tumor rate varying between 23 and 39%. The tumor age varied independently of the tumor rate; but the number of tumors being relatively small, not too much importance must be attached to variations in the tumor age. In the reciprocal crosses there are six strains. In three of those the tumor rate and tumor age were intermediate, but in two of these three the hybrids were nearer the English than the Cream. In the three other strains the hybrids followed the tumor rate of the Cream; in two of these, however, the tumors appeared earlier than in the Cream.

There are in addition two composite strains. In the first of these an English + Cream Hybrid with intermediate tumor rate was mated to a Cream. The admixture of the Cream reduced the tumor rate of the hybrid still farther. In the second composite hybrid the same hybrid strain was crossed with a hybrid strain with high tumor rate in which the English prevailed. In this the high tumor rate of the mother dominated.

Summary.—If a strain with a very high tumor rate (English) is crossed with a strain with a very low tumor rate (Cream), the hybrids show in seven out of ten instances an intermediate tumor rate. In three instances (50%) in which the mother strain was Cream, the tumor rate of the Cream prevailed, while this was found in no cross in which the mother was English and the father Cream. This again suggests the possibility that in certain instances the tumor rate of the mother strain may be of greater importance in determining the tumor rate than that of the father. Through further gradual addition of Cream to such hybrids the tumor rate was made to approach still more closely that of the Cream.

We see then that if we cross two strains with pronounced opposite tendencies, the result is in the majority of cases intermediate. While on the whole there is noticeable a certain correspondence between tumor rate and tumor age of these hybrids, certain independent variations of both seem to occur.

We shall now discuss crosses between Cream and 198 + (8 + German) F_4, between Cream and No. 10 Nov. 3 strain, and between Cream and European.

Cream + (198 + German Hybrid F_4) = 198 = English.

German Hybrid F_4 = 8 + German F_4. Two Black Cream males were crossed with two females. The record of these seven females was as follows:

Without tumors.	With tumors.
4 (3 II 1 III)	3 (2 I 1 II)

The old tumor rate of the mother strain was 63% (I age class), the later records showed a tumor rate of 43% (between I and II age classes).

The records of the hybrids are as follows:

Without tumors.	With tumors.
$F_1 - F_3$ 22 (5 I 15 II 2 III)	11 (5 I 6 II)
$66\frac{2}{3}$% (23% I 68% II 9% III)	$33\frac{1}{3}$% (45% I 55% II)

I age class: The tumor rate is intermediate, but nearer to the mother strain than to the Cream. The age class is that of the mother strain.

Cream + No. 10 (Nov. 3).

No. 10 (Nov. 3) = 151 (European + I daughter of No. 10 (Nov. 3)).

In the former cross of this kind the records were as follows:

Without tumors.	With tumors.
Former records:	
$F_1 - F_4$ 64% (16% I 29% II 55% III)	36% (6% I 37% II 57% III)
IV age class.	
$F_4 - F_6$, later records:	
74% (28% I 31% II 41% III)	26% (33% I 50% II 17% III)
I age class.	

The results were intermediate.

Two new crosses of the same kind were made in which different individuals were used.

(*a*) *White Cream + 697.*

White Cream was mated to three daughters of Tumor Mouse 697. No. 697 belonged to the No. 10 Nov. 3 strain. The records of these three females was as follows:

Without tumors	With tumors.
1 I 1 II	1 II

The cross behaved as follows:

Without tumors.	With tumors.
$F_1 - F_5$ 116 (47 I 41 II 28 III)	12 (2 I 4 II 6 III)
90½% (41% I 35% II 24% III)	9½% (16½% I 33½% II 50% III)
About IV age class.	

In this cross the Cream was dominant.

(b) White Cream + No. 10 (Nov. 3).

A White Cream male was mated to six No. 10 (Nov. 3) F_5 females which were offspring of the July, 1912, group. This is a cross which differs from the preceding one. The record of the original six females was as follows:

Without tumors.	With tumors.
2 I 1 II	2 I 1 II

The figures for the cross:

Without tumors.	With tumors.
98 (36 I 32 II 30 III)	51 (12 I 20 II 19 III)
66% (36½% I 32½% II 31% III)	34% (23% I 39% II 38% III)

The age class is between II and III.

The result in this hybrid is intermediate, just as it was in the previous crosses between Cream and No. 10 Nov. 3.

Cream + European.

(a) In one of our previous papers we reported on a cross between a Cream male and a European F_2 female which later became Tumor Mouse 428. All three daughters, which represented a cross between Cream and European, later developed tumors. In this case evidently the higher tumor rate of the mother strain was dominant. These daughters which later developed tumors were again mated to their Cream father.

Cream + (Cream + European 428) F_1.

Without tumors.	With tumors.
$F_1 - F_7$ 10 (3 I 6 II 1 III)	1 (1 II)
90% (30% I 60% II 10% III)	10% (100% II)

This cross resembles the Cream. Evidently the Cream father had a tendency to low tumor rate and the higher tumor rate of the Cream + European had been due to the influence of the European strain in the cross.

We produced a second hybrid strain between Cream male and European female; male Black Cream baby mice had been nursed by a tumor mouse of the G strain; one of these male Creams was mated to a European female. The offspring were very wild.

Without tumors.	With tumors.
$F_1 - F_4$ 70 (26 I 34 II 10 III)	34 (8 I 17 II 9 III)
68% (37% I 49% II 14% III)	32% (24% I 49% II 27% III)

The age class is between II and III, but nearer III.

In this case again the higher tumor rate of the European mother strain is dominant. In the tumor age there is apparently some influence of the Cream father strain noticeable.

We also made the reciprocal cross:

European + Cream.

A European F_7 male was mated to four Black Cream females. The four Black Cream females died without tumors, one in the I age period and three in the III age period. They behaved, therefore, like typical Creams.

The hybrids which were wild, similar to the Cream + European hybrids, had the following tumor rate:

Without tumors.	With tumors.
$F_1 - F_2$ 18 (10 I 5 II 3 III)	7 (1 I 3 II 3 III)
72% (55% I 28% II 17% III)	28% (14% I 43% II 43% III)

Again the higher tumor rate of the European is dominant over that of the Cream. The rate is similar to that of the reciprocal cross: Cream + European. The tumors belong, however, to the IV age class. Here the influence of the Cream is noticeable.

Summary.—We see, then, that in the crosses Cream + European, as well as in the reciprocal cross European + Cream, the higher tumor rate of the European dominates over the lower rate of the Cream. The tumor age is apparently influenced by the Cream component. If an additional Cream component is added to such a strain the tumor rate is lowered almost to the level of the Cream. In the cross between Cream + (198 + German Hybrid F_4) the result is intermediate; but on the whole the tumor rate is nearer that of the mother strain. The age class is that of the mother strain. In previous crosses between Cream and No. 10 (Nov. 3) the tumor rate had been intermediate. In a new cross of the same character in which new individuals entered, the tumor age was again intermediate and similar to the tumor rate of the earlier cross. The tumor age also was intermediate. In a second cross, however, the Cream tumor rate and tumor age were dominant. It is possible that in this case the three females which were sisters happened to transfer a tendency to a lower tumor rate.

German Hybrids.

We have previously reported on 8 + German Hybrids. The tumor rate in the old records is 41 per cent (II age class); in the intermediate and new records the tumor rate is 20 per cent (23 per cent), and the tumors appear somewhat earlier than those in the II age class. In one family of the later generations the tumor rate remained 43 per cent.

German + 8.

We now made the reciprocal cross: German + 8. One German male (about F_4) was mated to two No. 8 F_{12} females. These females died without tumors at the age of 12 months.

Without tumors.	With tumors.
$F_1 - F_4$ 112 (42 I 48 II 22 III)	0
100% (38% I 42% II 20% III)	0%

While in the 8 + German, the tumor rate of the German had been dominant, or at least very influential, we find in this strain a dominance of the No. 8. In the twelfth generation the tumor rate of the No. 8 had become very low (10%). We find here another instance in which apparently the mother strain is more influential in determining the tumor rate than the father strain.

Summary.—In the cross German + 8, we find dominance of the low tumor rate of the mother strain, while in the reciprocal cross 8 + German we find the higher tumor rate of the mother strain prevailing. In the two other crosses the higher tumor rates of the mother strains were dominant; the tumor age corresponded to that of the parents.

Waltzer Hybrids.

A series of hybridizations between waltzing mice, among which cancers are rare, and other strains, especially English, was carried out. In some crosses between Waltzer and English the high tumor rate of the English is almost dominant, while in the others the tumor rate is probably intermediate. The tumors always belong to the first age class, but they appear somewhat earlier in crosses which have a high tumor rate than in the others. In the crosses in which the English strain was almost dominant, further introduction of English made the record typically that of the English strain; while with the other hybrids, in which the combination with the Waltzer had considerably lowered the tumor rate of the English, the further intro-

duction of English did not raise the tumor rate of the crosses to a point where it was similar to that of the English; in this case the lower tumor rate of the father prevailed.

The crosses between Waltzer and Carter, Waltzer and No. 8, and (Waltzer + English) + (Cream + 10) either follow the tumor rate of both parents, or the higher tumor rate of the mother strain prevails.

Vermont Wild + (English) F_3.

A wild mouse captured in northern Vermont was mated to six English Sable (F_3) females, daughters of the Aug., 1911, group to which Tumor Mouse 437 belonged. These six English Sable mice died without tumors, two at the age of 7 months, two at the age of 10 months, one at the age of 13 months, one at the age of 14 months.

Without tumors.	With tumors.
$F_1 - F_4$ 241 (100 I 82 II 53 III)	138 (32 I 57 II 49 III)
63½ % (44% I 34% II 22% III)	36½% (23% I 41% II 36% III)

The tumor age is between the II and III age classes. The four generations behaved in a similar way.

While we do not know the tumor rate of the Vermont Wild mouse, it is at least probable that the tumor rate of these wild mice is low, perhaps comparable with that of the Cream. We would then have to conclude that the tumor rate of the hybrids is intermediate between that of both parent strains. The influence of the Vermont father strain is expressed in the relatively late tumor age, which differs so markedly from that of the English.

In a former similar experiment[1] we reported on the hybridization between a Michigan Wild mouse and English 101. In this case the tumor rate and tumor age of the crosses were approximately those of the English mother strain. We may conclude that in crosses between wild gray mice as the fathers and white mice as mothers, the result is probably either intermediate or the higher tumor rate and tumor age of the mother strain are dominant.

Parent with Higher Tumor Rate Is Dominant.

♀ indicates dominance of the mother strain.
♂ indicates dominance of the father strain.
The Roman figures indicate the age class of the offspring.
! indicates that importance can be attached to the results.
New records:
(*a*) Silver + English ♀ I !
(*b*) Silver + English ♀ I !
8½ + 328 ♀ I !
344 (English Sable) + German ♂.

English Sable + Cream Y ! ♂ (slightly below the English record, intermediate age class), also in intermediate record.
English Sable + (English Silver + I daughter of No. 10 Nov. 3 strain) ♂. The age class of the father dominates!
693 (English Tan + Cream) + 773 ($8\frac{1}{2}$ + 328) ♀. I age class !
Cream + (198 + German Hybrid F_4) ♀ (approaching intermediate) higher age class !
Cream + European ♀ (*a*) R !
Cream + European ♀ (*b*). Influence of Cream on tumor age R !
European + Cream ♂. Age class of Cream R !
8 + German ♀ (or intermediate) R !
(German + Carter) + (8 + German) ♀ (not much difference between the two parent strains).
(German + Carter) + (198 + German Hybrid F_4) F_3 (almost English tumor rate) ♀.
Waltzer + English (orange) (*a*) ♀. Age class of English.
(Waltzer + English (orange)) + English (orange) (*c*) ♀.

Complete English Tumor Rate and Age; Parent Strains with Similar Rate!

(Waltzer + English (orange)) + English Sable (*d*) ♀. English tumor age.
Walter + Carter ♀.
Waltzer + 8 ♀.
Old records:
Michigan Wild + English 101 ♀. English tumor rate.
English Sable (198) + ((103 + European) F_1 + III daughter of No. 10) ♂. Late tumors.
European 151 + I daughter of No. 10 ♀ !
European 151 + II daughter of No. 10 ♀ !
English + (8 + German) ♂ !
$8\frac{1}{2}$ + II daughter of No. 10 (not quite dominant) ♀ ! later decreasing tumor rate. Late tumors.
English Sable + (Silver + I daughter of No. 10) ♂ !
$8\frac{1}{2}$ + English Sable ♀. Early tumors !
English + (8 + German) ♂ !

Intermediate Tumor Rate.

New records:
344 (English Sable) +$8\frac{1}{2}$ F_4. Intermediate age class!
English Tan (121) + 3 Cream. Intermediate age class !
English Sable (344) + Black Cream. Intermediate age class !
English Sable + Cream Y. Intermediate age class (approaching tumor rate of English)!

693 (English Tan + Cream) + Cream !
Black Cream + English White. Intermediate age class !
Cream + English (Oct. 1913). Intermediate age class !
White Cream + White English. English age class !
Cream + English (1,031). Cream age class !
Cream + No. 10 (Nov. 3). Early tumors !
Cream + (198 + German Hybrid F_4) ♀. Early tumors (also in higher tumor rate records) !
White Cream + No. 10 (Nov. 3) (*b*). Intermediate tumor age.
Waltzer + English (white) (*b*). English age class.
Vermont Wild + English F_3 ♀. Late tumors.
Old records:
101 + (European + 103). Late tumors.
European + English Tan. Relatively late tumors!
Silver + I daughter of No. 10. Recently the tumor rate apparently increased and the tumor rate of No. 10 became almost dominating.
No. 8 + German.
Cream + No. 10 (Nov. 3) !

Parent with Lower Tumor Rate Is Dominant.

New records:
344 (English Sable) + $8\frac{1}{2}$ F_6 ♀. Earlier tumor age!
344 (English Sable) + 146 (European + English). The tumor rate in this cross was even lower than that of the mother strain ♀.
English Sable (4,444) + Cream ♀. Age class intermediate!
(*b*) 344 + Cream ♀. Age class intermediate!
(344 + Black Cream F_1) + Cream White ♀!
White Cream + 697 (No. 10 Nov. 3) (*a*) ♂. Cream age class.
Cream + (Cream + European 428) F_1 ♂ !
German + 8 ♀ R !
(Waltzer + English (white)) + English (*e*) ♂. English tumor age !
Old records:
London + (European + 103) F_3 ♀. Late tumor age.
German + 108 ♀ !

DISCUSSION.

In discussing these results we shall refer for comparison to some similar crosses we obtained in our previous hybridizations.

1. In crosses in which a male with a low, and a female with a high tumor rate are mated (Silver + English), the high tumor rate of the mother strain prevailed. In Silver + 10 the tumor rate was formerly

intermediate, but it increased more recently so that the higher rate of the mother became almost dominant. In English Sable + (Silver + I daughter of No. 10) the high tumor rate of the English prevailed throughout. We find, therefore, that in crosses between Silver, which is a low tumor rate strain, and high tumor rate strains in the majority of instances, the crosses follow the high tumor rate strains of the mother, or the results are intermediate. In these crosses the mother strain was the one with the higher tumor rate. In a cross of Silver with a strain of medium tumor rate (London) in which the Silver was the mother strain, the crosses showed an intermediate tumor rate, which, however, approached the Silver more closely than the London rate. There is, therefore, perhaps some indication in this case that the mother strain is more potent than the father strain. Individuals which themselves die without tumor, but belong to a strain with high tumor rate, may transmit to the offspring the tendency to a high tumor rate.

2. We may consider as control experiments those in which the tumor rates of both father and mother strains were similar to each other (high, medium, or low). In these cases the tumor rate of the offspring remained unchanged and resembled that of both parents.

3. While in two crosses, $8\frac{1}{2}$ + 328 (English Sable), the high tumor rate of the English dominated, and the influence of the mother is also noticeable in the tumor age, in the reciprocal crosses the tumor rate and tumor age of the $8\frac{1}{2}$ mother were much more influential. Some influence of the English in tumor rate (in one of the strains) and tumor age is, however, still noticeable. Again we have some indication that the mother strain is more influential than the father in determining the tumor rate. In a previous cross, $8\frac{1}{2}$ + II daughter of No. 10, the higher tumor rate of the mother strain prevailed; but later the tumor rate declined, in accordance with the decline in the tumor rate in No. 8 and $8\frac{1}{2}$.

4. The German Hybrids do not give decisive results, perhaps owing to the fact that the tumor rate of the German strain varied between high and medium and that this strain happened to be crossed with other strains of high or medium tumor rate.

In English Sable + German the higher tumor rate of the father probably prevailed, but in the tumor age an influence of the mother was noticeable.

Crosses No. 8 + German were at first intermediate, tending towards the higher tumor rate of the mother. Later the tumor rate fell in accordance with the fall in the tumor rate of No. 8. In a cross English + (8 + German) the higher tumor rate and the tumor age of the father prevailed. In the German + Carter the lower tumor rate of the mother strain prevailed. In German + 108 the lower tumor rate of the mother strain was probably more influential. When a German father was crossed with a lower tumor strain, the latter seemed to prevail. In two complex crosses in which German entered, the higher strain of the mother dominated.

5. While in 8 + German the higher tumor rate of the German was almost dominant, in the reciprocal cross the low rate of No. 8 prevailed. We find here another instance of the apparently greater influence of the mother strain in determining the tumor rate in the offspring.

6. While if we use different individuals as representatives of strains which are being hybridized, certain variations may occur in tumor rate and tumor age as indicated in two crosses between English Sable and European Hybrids, essentially the result was of a similar character in both cases in regard to tumor rate. The tumor age, however, followed in one instance the father, in the other the mother; and as in so many other cases a certain correspondence between tumor age and tumor rate was discernible.

7. In the crosses between English Sable and Cream the results were in all probability not due to accidental selection of unusual individuals. Experiments in which several individuals of a certain kind were used agreed with others in which only one male entered in the cross. Furthermore, the later records of English Sable 4,444 + Cream showed the same results as the earlier ones. The crosses between English and Cream are especially significant, because both parent strains differ markedly in their tumor rate and age. Both these strains have been carefully observed through a number of generations and their tumor rate and age were found constant in those substrains which were used for our experiments.

Seven out of ten crosses showed an intermediate tumor rate; in two of these the rate was nearer English than Cream, although the mother was Cream. In three instances in which the Cream was the

mother strain, the tumor rate of the Cream prevailed, while this did not occur in any of the reciprocal crosses. This again suggests the possibility that in certain cases the character of the mother strain may be of greater importance in determining the tumor rate than that of the father.

It is, furthermore, of interest that we find in the tumor rate of these crosses a range of variations from that approaching the tumor rate of the English to that of the Cream. Through a gradual further addition of Cream to such hybrids the tumor rate is made to approach still more closely that of the Cream. The tumor age also is mostly intermediate between that of the English and Cream, but may vary independently of tumor rate either in one direction or the other.

8. In crosses between Cream and 198 + German Hybrid F_4, in which the Cream was the father, the high tumor rate and the early tumor age of the mother strain were more potent than the tumor age and rate of the Cream, while of three crosses between Cream and No. 10, two gave an intermediate tumor rate and in one instance the low tumor rate of the Cream was dominant. In the latter case the tumor age of the Cream was likewise dominant, while in the former crosses the tumor age was intermediate.

We see then (1) that a certain correspondence exists between the tumor age and the tumor rate of the various crosses, the lower tumor rate being associated with later tumors, and (2) that crosses between the same strains in which different individuals are used, may show certain variations in tumor rate and tumor age among each other. In two crosses between Cream and European, as well as in one reciprocal cross between European and Cream, the European component influenced the tumor rate to a marked degree and was probably dominant. Cream, however, exerted a certain influence on the tumor age. In the reciprocal cross European + Cream, in which the Cream females behaved like typical Creams, the European again greatly influenced the tumor rate, although the tumor age was that of the Cream. As in the preceding cross, we see a certain dissociation between tumor age and tumor rate in the hybrids. The reciprocal crosses gave similar results. The combination of Cream + European female with a Cream male led to a close approximation of the Cream records.

If we now consider all the hybrids in which Cream entered, we find the following results:

Cream: intermediate results 12 (1 of these contained 2 Cream components). Omitting the latter hybrids, intermediate results 11. In 8 of these the Cream was the father and in 4 crosses Cream was the mother.

Cream prevailed: 5 (2 of these contained 2 Cream components). Omitting the latter, Cream dominated in 3 cases. In 3 of the 5 crosses the Cream was mother and in 2 it was father.

The strain associated with Cream was dominant: 5.

Two of these stood between this group and the intermediate and were also included among the intermediate. Excluding these two crosses, three kinds of hybrids belong to this group. It is possible that these strains will have to be classed as intermediate and that European was not completely dominant in these cases.

In three of these the Cream was father and in two the Cream was mother. We see, then, that in a large majority Cream crossed with a high tumor rate strain produces crosses with an intermediate tumor rate. In a few instances the Cream is dominant; and in a few cases the other strain with the higher tumor rate may be dominant.

9. The crosses in which Waltzer constitutes one of the parent strains suffer from lack of definite knowledge as to the exact tumor rate of the Waltzer strain. We know that tumors of the breast occur in waltzing mice; but as far as we were able to ascertain, tumors among the strains of waltzing mice which we used for hybridization were rare and presumably did not greatly differ from the tumor rate of the Cream. While their tumor rate may have been somewhat higher than that of the Cream they certainly did not represent a high tumor rate strain. Crossed with an English (orange) a tumor rate and tumor age appeared which approached those of the English mother. When these crosses were again hybridized with various English females, complete tumor rate and tumor age of the English mother were obtained. On the other hand, a cross between a male Waltzer and another English female, which herself developed a tumor, gave much lower tumor rate. It was probably intermediate between that of both parents. In a further cross between these latter hybrids and English the lower tumor rate of the father strain prevailed, but the tumors belonged to the first age class. We see then that, in this case, the Waltzer crossed with English imposed its tumor rate with such an intensity that this

tumor rate held its own in further hybridizations of the cross with English. In a similar way in crosses in which (European + 103) entered, this hybrid strain had a general tendency to transmit lateness of the tumors to the offspring. In this instance the influence of the Waltzer in the cross which served as the father strain was so marked that it dominated in a second hybridization with an English; we see, therefore, that the lower tumor rate of the father may prevail even against the high tumor rate of an English mother. But in all these hybridizations the tumor age was very early and was probably impressed upon the offspring by the English component. In the crosses between Waltzer + Carter and Waltzer + 8 the higher tumor rate of the mother strain was in all probability dominant.

10. The same reservation which we made in the case of the Waltzer applies also in the case of the Vermont Wild mouse which was mated to an English female. We may assume that tumors are rare and appear late in this wild strain and that the Vermont strain probably resembles the Cream mice. The hybrids probably presented an intermediate tumor rate; the tumors were relatively late. We can be certain that neither the English tumor rate nor age dominated. In a previous experiment, however, in which a Michigan Wild mouse was mated to an English female, the English tumor rate and age prevailed. In accordance with what we have observed in the other cases, we may interpret these results as indicating that if we cross a high tumor rate strain with a low tumor rate strain, the results may sometimes be intermediate, but that at other times the high tumor rate strain may dominate.

Effect of Sex of Parents on the Tumor Rate of the Hybrids.

The parent with higher tumor rate is dominant in 24 strains.
The mother strain dominates in 17 strains.
The father strain dominates in 7 strains.
The tumor rate is intermediate in 19 strains.

Parent with lower tumor rate is dominant in 10 strains.
The mother strain dominates in 8 strains.
The father strain dominates in 2 strains.
Only in approximately 18 per cent of the strains does the lower tumor rate prevail.
Altogether in 25 crosses the mother strain dominated, and in 9 crosses the father strain dominated.
In 19 strains the result was intermediate.

We may therefore conclude that both mother strain and father strain may prevail; but the mother strain dominated in a much larger number of our cases than the father strain. This fact, taken with several observations in which in reciprocal crosses the hybrids followed the tumor rate of the mother strain, suggests that the mother strain may possibly be of greater significance in determining the tumor rate in cancer of the mammary gland than the father strain. While the tumor rate is certainly not altogether a sex-linked character, there may perhaps exist a partial linking of the determiners of the tumor rate with the sex determinant.

SUMMARY AND CONCLUSIONS.

1. In order to test our previous conclusions concerning the tumor rate in hybrid strains, we carried out additional hybridizations. For this we selected strains which differed markedly in their tumor rate and which had been followed through a number of generations and found constant in their behavior.

Hybridizations between strains or families of a similar (either high, medium, or low) tumor rate served as control experiments. In these cases the offspring showed a tumor rate similar to that of the parents.

2. In selecting for hybridization various groups of Cream mice representing a very low tumor strain and English Sable mice representing a high tumor strain, we obtained in the majority of cases hybrid strains with a tumor rate intermediate between that of both parent strains. In a few instances it approached somewhat the high tumor strain of the English, and in a few others the low tumor rate of the Cream.

3. In several hybrids between the high tumor strain English and the low tumor strain Silver, the latter of which was split off from the English, the high tumor rate of the English prevailed. A number of mice which served for hybridization were followed throughout their life and found to behave typically as to tumor rate.

4. If we omit the strains in which both parents had a similar tumor rate, we found the higher tumor rate to be dominant in twenty-four hybrids. In seventeen of these the mother strain dominated, and in seven the father strain. In nineteen hybrid strains the tumor

rate was intermediate. In ten strains the lower tumor rate dominated. In eight of these the mother strain prevailed and in two the father strain. The low tumor rate was, therefore, dominant in approximately 18 per cent of the strains.

5. There does not seem to be a fixed rule as to dominance in the tumor rate. In a considerable number of cases, and especially in well analyzed cases, the result was intermediate.

6. Altogether in twenty-five of our hybrid strains the mother strain, and in nine strains the father strain prevailed. In nineteen strains the result was intermediate. We conclude that both father and mother strain may dominate and that the tumor rate is not in the strict sense of the term a sex-linked character. However, the fact that the mother strain prevailed in a much larger number of our cases than the father strain, and that several times (but not in all cases), in reciprocal crosses, the hybrids followed the tumor rate of the mother strain, suggests the possibility that as far as the hereditary transmission of mammary cancer in mice is concerned, the mother may be more potent than the father, and that perhaps under certain quantitatively varying conditions the mother strain may dominate over the father strain. This statement is merely a tentative conclusion at the present time and needs further investigation.

7. The results of these investigations confirm our previous conclusion that in the majority of the crosses which we observed, the cancer rate is either intermediate between those of father and mother strain, or that it follows the tumor rate of the parent with the higher rate and only in a relatively small number of instances the cancer rate follows that of the parent strain with the lower tumor rate. On the whole, the heredity of cancer rate and cancer age follows the blending type of hereditary transmission.

8. While there is a distinct relation between high tumor rate and early cancer age, our observations make it probable that cancer rate and cancer age are to some extent independent of each other.

9. On the whole the different generations, including F_1 and F_2 of the various hybrid strains, showed a concordant tumor rate and tumor age.

10. If we consider, then, our results as a whole, we may conclude that in crossing strains which differ in their tumor rate no rule of domi-

nance which applies equally to all cases seems to exist. In a certain number of crosses the results are undoubtedly intermediate. In these instances the tumor rate and to some extent also the tumor age behaves in a manner similar to characters which differ in father and mother merely in quantity as in the length of organs. From such intermediate results all kinds of gradations exist, leading on one side to dominance of the strain with the higher tumor rate and on the other side to dominance of the strain with the lower tumor rate. However, in our experiments dominance of the strains with the higher tumor rate greatly predominated over the opposite extreme.

11. Our results on the whole are, therefore, in certain respects comparable with the inheritance of sex which Goldschmidt studied in hybrid strains of the gypsy mother. Here also all gradations from the male to the female were observed in the offspring. Goldschmidt assumes that in different hybrids there are created different quantities of certain substances which like enzymes determine according to their quantity the velocity of chemical reactions and the amount of certain substances produced. The latter determine in the hybrids the quantitative variations in the character which is analyzed. According to Goldschmidt, multiple allelomorphs, which in our experiments seem to determine the heredity of spontaneous cancer, depend upon differences in the quantity of a substance present in the different individuals or varieties. In whatever way we may conceive of the character of multiple allelomorphs, our results make it probable that multiple factors are involved in the heredity of cancer in mice.

THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND THE EFFECT ON RENAL FUNCTION OF CHANGES IN THIS EQUILIBRIUM.

I. A Study of the Acid-Base Equilibrium of the Blood in Naturally Nephropathic Animals and of the Functional Capacity of the Kidney in Such Animals Following an Anesthetic.*

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Plates 49 and 50.

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As a result of the observations of Ophüls,[1] Pearce,[2] and Dayton,[3] the fact is generally known that many of the lower animals, particularly the dog, are susceptible to a type of kidney injury which should be classed as a chronic nephropathy. In a recent study[4] of the naturally acquired chronic nephropathy of the dog these earlier observations have been confirmed, the various nephropathic processes have been classified, and a consideration of the processes of repair in the kidneys has been undertaken. In this study of forty-two naturally nephropathic animals I found it possible, with three exceptions, to classify the kidney injury as a chronic productive type. The three remaining animals showed the typical arteriosclerotic type of kidney with extensive general sclerosis of the vessels. The thoracic aorta in one of the animals was the seat of a fusiform aneurysm. In the majority of kidneys of the remaining thirty-nine animals the

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formation of connective tissue was a focal process confined to the glomeruli. In all the animals both the capsule and capillaries of the glomeruli participated in the laying down of connective tissue so that in the different animals it was not possible to specialize the glomerular pathology into a capsular and intracapillary glomerulonephropathy. Hyaline degeneration of the fibrosed capillary tufts was occasionally observed.

The formation of intertubular connective tissue in the kidneys of these animals has shown no parallel with the degree of fibrosis which has taken place in the glomeruli, and, furthermore, there has existed a notable disproportion between the severity of the changes in the glomeruli and the degree of degeneration of the tubular epithelium. This observation has been recently confirmed by Stengel, Austin, and Jonas[5] in a study of the chronic nephropathies in human material.

The above outline of the naturally acquired chronic nephropathy of the dog not only establishes the frequency of the occurrence of such conditions in these animals but shows the close histological resemblance between certain nephropathies of the lower animals and man.

The ability to obtain such material for experimental purposes offers many possibilities for the study of the chronic nephropathies and for the study of various acute processes which may be superimposed upon the naturally acquired chronic kidney injury.

The first study[6] of this character has consisted in an investigation of the functional response of the naturally nephropathic kidney after the kidney had been acutely injured by uranium nitrate or by an anesthetic. An analysis of these experiments shows that when a naturally nephropathic animal is anesthetized by Gréhant's anesthetic, or when the animal is given uranium and anesthetized by ether, the animals fall into two clearly defined groups. One group of animals during the anesthesia becomes rapidly anuric and fails to show a functional response to such diuretic substances as theobromine, caffeine, and solutions of urea and glucose. The second group of animals remains diuretic following the anesthetic and shows a functional response to the diuretic substances which in the first group of animals were of no diuretic value.

A physiological study of the response of the vascular mechanism of the kidney in the anuric and diuretic groups of animals by the use of such peripherally acting stimuli as the members of the caffeine group and adrenalin has shown this

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[5] Stengel, A., Austin, J. H., and Jonas, L., A comparison of the functional and anatomic findings in a series of cases of renal disease, *Arch. Int. Med.*, 1918, xxi, 313.

[6] MacNider, A pathological study of the naturally nephropathic kidney of the dog, rendered acutely nephropathic by uranium or by an anesthetic. Part II, *J. Med. Research*, 1916, xxxiv, 199.

mechanism to be responsive in both groups of animals. The degree of vasodilation or constriction of the renal vessels induced by caffeine or adrenalin was usually greater in the anuric than in the diuretic groups of animals. Renal vasodilation effected by caffeine, theobromine, or solutions of urea or glucose in the diuretic group of animals was associated with a free diuresis, while with the production of an even greater degree of vasodilation by these substances in the anuric animals no formation of urine was induced.

The histological study of the kidneys of these two groups of animals has shown a chronic productive nephropathy in which the changes were largely confined to the glomeruli. As a result of the use of uranium or an anesthetic no acute degenerative changes had developed in the glomeruli. The vascular pathology in the two groups has shown no elements of difference. The acutely developing pathological change which differentiates the anuric from the diuretic groups of animals has consisted in the degree of degeneration occurring in the tubular epithelium, and especially in the epithelium of the convoluted tubules. The animals which have remained diuretic and responsive to diuretic substances have shown but slight epithelial damage, while the group of animals that have been rendered anuric by the anesthetic and non-responsive to the same diuretic substances have shown a swelling and necrosis of the convoluted tubule epithelium. The ascending limbs of Henle's loops have contained a large amount of stainable fat.

The following investigation has been undertaken with the object in view of ascertaining the difference in the response of the normal and naturally nephropathic kidney to Gréhant's anesthetic, the principal anesthetic ingredient of which is chloroform. The study embraces an investigation of the acid-base equilibrium of the blood in these two groups of animals, prior to and during the period of anesthesia, and the association of the changes in this equilibrium with the development of an anuria. The functional capacity of the kidney has been determined by the phenolsulfonephthalein test, the retention of blood urea, and the response of the kidney during the period of anesthesia to various diuretic substances. Finally, the relative toxicity of this anesthetic for the normal as compared with the naturally nephropathic kidney has been investigated by a histological study of the kidneys at the termination of the experiments.

EXPERIMENTAL.

Dogs were employed in the experiments which furnish the basis for this study. Nine of the animals were healthy dogs varying in age from 8 months to 6 years and 2 months. Eighteen of the dogs were naturally nephropathic and varied in age from 3 years to 13 years and 1 month. Both the normal animals which served as controls and the naturally nephropathic animals were placed in metabolism cages, given 500 cc. of water daily, and fed on bread with a small amount of cooked meat. The animals were studied for 3 days prior to the day of experiment. During this period the urine was collected twice a day and examined qualitatively for albumin, glucose, acetone, and diacetic acid. Quantitative determinations of these substances were made when present. The centrifugalized urine was examined for casts. The hydrogen ion content of the blood was determined by the method of Levy, Rowntree, and Marriott,[7] the alkali reserve of the blood and the tension of carbon dioxide in alveolar air by the methods of Marriott,[8,9] while the blood urea was determined by the method of Marshall[10] as modified by Van Slyke and Cullen.[11] The phenolsulfonephthalein test for kidney function was conducted according to the technique of Rowntree and Geraghty.[12]

At the end of the 3 day period allowed for normal observations the animals were given 300 cc. of water and 3 hours later were given 60 cc. per kilo of Gréhant's anesthetic by stomach tube. Half an hour was allowed for the development of a degree of anesthesia sufficient for

[7] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., A simple method for determining variations in the hydrogen-ion concentration of the blood, *Arch. Int. Med.*, 1915, xvi, 389.

[8] Marriott, W. McK., A method for the determination of the alkali reserve of the blood plasma, *Arch. Int. Med.*, 1916, xvii, 840.

[9] Marriott, The determination of alveolar carbon dioxid tension by a simple method, *J. Am. Med. Assn.*, 1916, lxvi, 1594.

[10] Marshall, E. K., Jr., A rapid clinical method for the estimation of urea in urine, *J. Biol. Chem.*, 1913, xiv, 283.

[11] Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix, 211.

[12] Rowntree, L. G., and Geraghty, J. T., An experimental and clinical study of the functional activity of the kidneys by means of phenolsulphonephthalein *J. Pharmacol. and Exp. Therap.*, 1909–10, i, 579.

the surgical part of the experiment. The first observations were made on the anesthetized animals 1 hour after they had received the anesthetic and half an hour after the development of a satisfactory state of general anesthesia. At half hour periods during the course of the experiments the flow of urine per minute was recorded and the hydrogen ion content and reserve alkali of the blood were determined. At these intervals the animals were given intravenously one of the following diuretic substances: caffeine citrate or theobromine sodium salicylate in 1 per cent solution, 1 cc. per kilo; pituitrin (Parke, Davis and Company) 0.5 cc.; or solutions of urea or glucose. The urea solution was of 0.9 per cent strength in 0.9 per cent sodium chloride and was given in the quantity of 10 cc. per kilo. The glucose solution was 20 per cent strength in 0.9 per cent sodium chloride and was given in the same quantity per kilo as was the urea solution.

The experiments on the anesthetized animals were terminated at the end of $1\frac{1}{2}$ hours at which time the tension of carbon dioxide in alveolar air was determined and the kidneys were removed for the histological study.

Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.

The following observations which have extended over a period of 3 days have been recorded in Table I. In this table observations on two normal or control animals have been tabulated with similar observations on nine of the naturally nephropathic animals.

In recent papers[13, 14] studies have been made of the relative stability of the acid-base equilibrium of the blood in animals of different ages. In these studies it was found that the older animals were more susceptible to agents which altered this equilibrium in favor of the acid ion than were the younger animals. For this reason relatively young animals were selected for the controls in these experiments. The factor of the age of the animal as expressed by the ease with which

[13] MacNider, A consideration of the relative toxicity of uranium nitrate for animals of different ages. I, *J. Exp. Med.*, 1917, xxvi, 1.

[14] MacNider, Concerning the influence of the age of an organism in maintaining its acid-base equilibrium, *Science*, 1917, xlvi, 643.

the nephropathic animals develop an acid intoxication cannot be accurately determined in these experiments, since the existence of the chronic nephropathy in the animals, as will be demonstrated later in this study, makes them more susceptible to changes in the acid-base equilibrium. The naturally nephropathic animals have, however, been arranged in the table according to their age. The influence of this factor in determining the toxicity of the anesthetic will be referred to in a later part of this paper.

Reference to Table I shows the control animals to be freely diuretic and the urine to be free from albumin, glucose, acetone bodies, and casts. The hydrogen ion content of the blood has varied from 7.3 to 7.45, while the reserve alkali has shown a variation from 8 to 8.1. The hydrogen ion determinations by the method employed in these experiments do not show a correlation with the reserve alkali determinations or the determinations of alveolar air carbon dioxide. The Levy-Rowntree-Marriott method is an expression of both the volatile and non-volatile acid content of the blood. Even a local accumulation of carbon dioxide in the blood of a part, such as the arm or leg, may give a very high reading and is not a true expression of the non-volatile acid content of the blood. For this reason the alkali reserve determinations and the variations in alveolar air carbon dioxide tension more accurately indicate the changes in the hydrogen ion content.

The determinations of alveolar air carbon dioxide for the normal animals have varied between 37 and 40 mm. and have shown a correlation with the maximum and minimum variations of the reserve alkali of the blood.

The blood urea estimations have remained very constant. In one of the nine control animals the percentage of urea was 0.014 per cent, while in the remaining animals the blood urea was 0.012 per cent. The phenolsulfonephthalein test was made on six of the control animals. The total output of the dye in a 2 hour period varied from a minimum of 73 per cent to a maximum of 81 per cent.

A study of the observations on the naturally nephropathic animals prior to an anesthetic (Table I) shows a marked variation in the output of urine by the different animals in a 24 hour period. This has varied in the respective animals from 174 to 820 cc. The urine of all the animals contained albumin and casts. In all but two of the animals

TABLE I.

Observations on Normal and Naturally Nephropathic Animals Prior to an Anesthetic.

Experiment No.	Age of animal.	Weight.	Water in 24 hrs.	Urine in 24 hrs.	Albumin and casts.	Acetone and diacetic acid.	pH	R. pH	Carbon dioxide.	Blood urea.	Phenolsulfonephthalein.
	yrs.	*kg.*	*cc.*	*cc.*					*mm.*	*per cent*	*per cent*
1 (control).	1	8.2	500	481	0	0	7.3	8.0	37	0.012	
2 (control).	3	13.66	500	287	0	0	7.45	8.1	40	0.012	81
3	1–2	11.3	500	174	Tr. Casts.	0	7.45	8.15	40	0.015	68
4	1–2	21.53	500	518	" "	0	7.45	8.1	39		
5	1–2	7.32	500	389	1.2 gm. Numerous casts.	0	7.45	7.9	36	0.018	54
6	3 yrs. and 4 mos.	11.4	500	584	Tr. Casts.	0	7.4	8.0	40		
7	8	17.7	500	820	" "	0	7.45	8.1	40		
8	10	23.1	500	508	" "	0	7.45	8.0	38		
9	10–11	21.55	500	355	" Numerous casts.	0	7.35	8.0	38	0.015	61
10	12	23.4	500	718	" Few casts.	0	7.45	8.0	41	0.015	69
11	13 yrs. and 1 mo.	30.35	500	520	0.9 gm. Few casts.	0	7.45	8.1	32	0.028	52

albumin was present as a mere trace. In one animal the urine contained 1.2 gm. of albumin per liter. This animal (Experiment 5, Table I) as contrasted with the other naturally nephropathic animals showed a depletion in the alkali reserve of the blood, a decrease in the tension of alveolar air carbon dioxide, and a marked reduction in the elimination of phenolsulfonephthalein. None of the naturally nephropathic animals of this series has shown the presence of acetone bodies in the urine. The hydrogen ion determinations have been variable as was the case with the control animals. With one exception (Experiment 5, Table I) the alkali reserve of the blood and the tension of alveolar air carbon dioxide in the naturally nephropathic animals have been within the range of normality. The reserve alkali in these animals has varied from 8 to 8.1, while the carbon dioxide tension has varied between 32 and 41 mm. This minimum variation of 32 mm. in carbon dioxide tension does not correlate with the reserve alkali reading for the blood which was 8.1. The question arises as to whether or not some local pathology in the lung of this very old animal could not have been responsible for this atypical reading.

The blood urea determinations have shown a retention in all the naturally nephropathic animals, varying from 0.015 to 0.028 per cent. The elimination of phenolsulfonephthalein has been reduced in all the animals and, as is shown in Table I, there is a relation between the retention of blood urea and the elimination of phenolsulfonephthalein. The animal of Experiment 11, with the greatest retention of blood urea, 0.029 per cent, also shows the greatest reduction in the output of phenolsulfonephthalein which was 52 per cent. The animals with a lower percentage retention of blood urea have a higher percentage elimination of the dye.

From the foregoing analysis of observations on the control and naturally nephropathic animals prior to the use of an anesthetic, the following conclusions are permissible: (1) The control animals show no evidence of a kidney injury and have a normal acid-base equilibrium. (2) The naturally nephropathic animals give evidence of a chronic kidney injury by the formation of a variable amount of urine which contains albumin and casts, by a retention of blood urea, and by a decrease in the elimination of phenolsulfonephthalein. (3) The naturally nephropathic animals with one exception, Experiment 5,

show a normal acid-base equilibrium. (4) In the naturally acquired chronic nephropathy of the dog in which the chronic pathology is largely confined to the glomeruli an acid intoxication is not the primary cause for the kidney injury. (5) In such chronic nephropathies blood urea determinations and estimations of the ability of the kidney to eliminate phenolsulfonephthalein are of more diagnostic value than determinations of the acid-base equilibrium of the blood.

Observations on Normal and Naturally Nephropathic Animals after an Anesthetic.

An analysis of the response of the control animals to Gréhant's anesthetic as indicated in Table II shows these animals to have remained diuretic following the development of a state of surgical anesthesia. The urine flow varied between 1 and 2 drops per minute for both animals. Half an hour after the establishment of a state of anesthesia the urine flow was unaffected and no change from the normal alkali reserve reading of 8 to 8.1 had occurred. During the remaining hour of the experiment these animals were freely diuretic to theobromine, caffeine, pituitrin, and a solution of glucose. The flow of urine from pituitrin was increased from 5 to 20 drops per minute and an even greater diuretic effect was obtained from the glucose solution, the urine increasing from 5 to 26 drops per minute.

During the course of the experiments the control animals were able to maintain their normal acid-base equilibrium. The alkali reserve of the blood failed to show any depletion and the tension of carbon dioxide in alveolar air remained practically unaffected. At the end of the experiment the control animals were forming a larger amount of urine than was the case at the commencement of the anesthesia. At the commencement of the experiments the urine flow per minute for the animals of Experiments 1 and 2 was 2 and 1 drops for the respective animals, while at the termination of the experiments the urine flow was 3 and 6 drops per minute for these animals.

The histological study of the kidneys of the control animals has been negative in as far as demonstrating any pathological change induced by the anesthetic. The glomerular vessels are distended with blood and the capillary loops usually fill the capsular space. The tubular epithelium, and especially that of the convoluted tubules,

is shrunken, the nucleus-plasma relationship has increased in favor of the nucleus, and the nuclei are hyperchromatic and stain intensely. The ascending limbs of Henle's loops either contain no stainable fat or a very small amount of fat in the form of dust-like particles.

A study of the response of the naturally nephropathic animals to Gréhant's anesthetic given in the same quantity per kilo as was the case with the control animals shows (with two exceptions, Experiments 4 and 6, Table II) that all the naturally nephropathic animals were rendered anuric by the anesthetic in ½ hour after the anesthetic was administered. At this period in the experiments the table shows that the anesthetic had induced a rapid depletion in the alkali reserve of all the naturally nephropathic animals which had become acutely anuric, while in the animals which at this period remained diuretic the alkali reserve had either undergone no change from the normal reading prior to the anesthetic or the reduction in the alkali reserve was not below 8.05. For example, the naturally nephropathic animal of Experiment 4 remained diuretic following the anesthetic and showed only a slight variation in the alkali reserve reading, 8.1 to 8.05. The animal of Experiment 6, which also remained diuretic, showed no change in the alkali reserve. The reading was 8 before and after the development of an anesthesia. The naturally nephropathic animals which became rapidly anuric show a depletion in the alkali reserve. The animal of Experiment 3 showed a reduction in the reserve alkali from 8.15 to 8, while in the animal of Experiment 8 the reserve alkali was reduced by the anesthetic from a normal reading of 8 to 7.9.

At this period of the experiments, the end of the first half hour, the functional response of the kidney was investigated by giving the animals caffeine, theobromine, or pituitrin. A study of Table II shows these substances, in the animals in which the anesthetic had induced a rapid depletion of the alkali reserve of the blood, to be of no diuretic value. The animals remained anuric. In the animal of Experiment 6, in which there had occurred no change in the alkali reserve from the normal reading, theobromine induced a free diuresis, the output of urine increasing from 2 to 10 drops per minute. In the animal of Experiment 4 in which the anesthetic had brought about a reduction in the alkali reserve from 8.1 to 8.05 caffeine was of no diuretic value.

At the end of the 1st hour of the anesthesia all the naturally nephropathic animals had become anuric. The alkali reserve of the blood had been reduced to 7.9 in all the animals except the dog of Experiment 10. The reserve alkali reading for this animal was 7.8. At this period of the experiment the functional response of the kidney was again tested by employing as diuretics theobromine, or solutions of urea and glucose. The kidneys of the naturally nephropathic animals were non-responsive to these substances which in the control animals had induced a marked diuretic effect. The anuria which has been associated with the development of an acid intoxication on the part of the anesthetized naturally nephropathic animals was unaffected by these diuretic solutions.

The experiments were continued for the third half hour period. During this time the animals remained anuric and the reserve alkali of the blood showed a progressive decrease in all the animals. At the termination of the experiments, 1½ hours after the first observations had been made, the reserve alkali readings for all the naturally nephropathic animals varied from a maximum reading of 7.85 to the extremely low reading of 7.45. The determinations of carbon dioxide tension in alveolar air at the close of the experiments varied between 22 and 10 mm. and showed the usual correlation with determinations of the alkali reserve of the blood.

In view of the previously mentioned observation that old animals were more susceptible to agents which induced an acid intoxication than were young animals, it is interesting to note that the two oldest naturally nephropathic animals gave evidence of having developed the severest acid intoxication. The animal of Experiment 10, 12 years old, had at the termination of the experiment an alkali reserve of 7.6 and a tension of alveolar air carbon dioxide of 18 mm., while the animal of Experiment 11, 13 years and 1 month old, had a reserve alkali of only 7.45 and a tension of alveolar air carbon dioxide of 10 mm.

The histological study of the kidneys of the naturally nephropathic animals after the establishment of an anuria by Gréhant's anesthetic has not shown any acute degenerative change or other evidence of vascular injury to the glomeruli. The capillary tufts usually fill the capsular space unless their distention has been prevented by a forma-

TABLE II.

Toxic Effect of an Anesthetic on the Functional Capacity of Normal and Naturally Nephropathic Kidneys.

Experiment No.	Anesthetic (Gréhant's).	Urine per min.	pH ½ hr. after anesthetic.	R. pH ½ hr. after anesthetic.	Urine per min.	Diuretic.	Urine per min.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Diuretic.	Urine per min.	pH 1½ hrs. after anesthetic.	R. pH 1½ hrs. after anesthetic.	Carbon dioxide 1½ hrs. after anesthetic.	Urine per min.	Fat in renal epithelium.
	per cent	*gtt.*			*gtt.*		*gtt.*				*gtt.*			*mm.*	*gtt.*	
1 (control).	60	2	7.45	8.0	2	Theobromine 1%.	5	7.45	8.0	Pituitrin 0.5 cc.	21	7.35	8.0	36	3	Tr.
2 (control).	60	1	7.4	8.1	2	Caffeine 1%.	5	7.4	8.1	Glucose sol. 20%.	26	7.4	8.1	39	6	"
3	60	0	7.45	8.0	0	Pituitrin 0.5 cc.	0	7.4	7.9	Urea sol. 0.9%.	0	7.1	7.8	19	0	L.*
4	60	2	7.4	8.05	0	Caffeine 1%.	0	7.3	7.9	" " 0.9%.	0	7.25	7.85	22	0	"
5	60	0	7.45	7.8	0	Theobromine 1%.	0	Not made.	Not made.	Glucose sol. 20%.	0	7.35	7.85	Not made.	0	"
6	60	2	7.35	8.0	2	Theobromine 1%.	10	7.4	7.9	Theobromine 1%.	0	7.25	7.85	21	0	"
7	60	0	7.3	8.0	0	Caffeine 1%.	0	7.35	7.9	Theobromine 1%.	0	7.2	7.8	20	0	"
8	60	0	7.4	7.9	0	Theobromine 1%.	0	7.4	7.9	Theobromine 1%.	0	7.3	7.85	20	0	"
9	60	0	7.2	7.9	0	Caffeine 1%.	0	7.4	7.9	Urea sol. 0.9%.	0	7.35	7.8	21	0	"
10	60	0	7.35	7.9	0	Theobromine 1%.	0	7.4	7.8	Theobromine 1%.	0	7.3	7.6	18	0	V. L.
11	60	0	7.4	8.0	0	Pituitrin 0.5 cc.	0	7.35	7.9	Urea sol. 0.9%.	0	7.15	7.45	10	0	"

*L, indicates large amount; V. L., very large amount.

tion of connective tissue. The characteristic and constant change which is induced by the anesthetic is an acute swelling, vacuolation, and necrosis of the epithelium of the convoluted tubules, and a rapid accumulation of stainable fat in the ascending limbs of Henle's loops. Fatty degeneration of a slight degree is occasionally seen in the degenerating convoluted tubule epithelium. The amount of stainable fat in the epithelium has shown a relation with the degree of acid intoxication in the various animals. The animals which have shown the greatest depletion in their alkali reserve and the lowest tension of carbon dioxide in alveolar air have also shown the greatest accumulation of fat in the degenerated epithelium, especially the epithelium of Henle's loops (Figs. 1 and 2).

DISCUSSION.

During the past 10 years numerous investigations have been concerned with the occurrence and significance of an acid intoxication in the acute and chronic nephropathies. As early as 1888 von Jaksch[15] noted a decrease in the alkalinity of the blood in uremia and this observation was confirmed in 1898 by Brandenburg.[16] Von Hösslin[17] in 1909 noted in certain of the nephropathies a definite relation between the acidity of the urine and the amount of albumin and number of casts, and in a later paper[18] after observing that very large amounts of alkali were necessary to reduce the acidity of the urine in nephritics, recommended rather indiscriminately the use of an alkali as a therapeutic measure. In 1912 Sellards,[19] employing his alkaline tolerance test in a group of nephropathies, noted a retention of bicarbonate in the acute nephropathies with uremia, and at

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[15] von Jaksch, R., Ueber die Alkalescenz des Blutes bei Krankheiten, *Z. klin. Med.*, 1888, xiii, 350.

[16] Brandenburg, K., Ueber die Alkalescenz des Blutes, *Z. klin. Med.*, 1899, xxxvi, 267.

[17] von Hösslin, R., Ueber die Abhängigkeit der Albuminurie vom Säuregehalt des Urins, *Münch. med. Woch.*, 1909, lvi, 1673.

[18] von Hösslin, Über die Abhängigkeit der Albuminurie vom Säuregrad des Urins und über den Einfluss der Alkalizufuhr auf Acidität, Albuminurie, Diurese und Chloridausscheidung, sowie auf das Harnammoniak, *Deutsch. Arch. klin. Med.*, 1912, cv, 147.

[19] Sellards, A. W., The determination of the equilibrium in the human body between acids and bases with especial reference to acidosis and nephropathies, *Bull. Johns Hopkins Hosp.*, 1912, xxiii, 289.

about the same time Porges and Leimdörfer,[20] using determinations of carbon dioxide tension as an index of an acid intoxication, concluded that there occurred in general a reduction of carbon dioxide tension parallel to the symptoms of uremia. The work of Straub and Schlayer[21] which was confirmed by Barcroft[22] and his pupils has apparently established the fact that in the type of acute kidney injury characterized by the symptom complex uremia, there is a direct connection between the uremic manifestations and an acid intoxication.

The question which is still undecided is concerned with the association of an acid intoxication in those acute and chronic nephropathies which have not developed symptoms of uremia and whether or not when such an intoxication occurs in these cases it should be considered as a retention acidosis due to the kidney injury or whether it should be looked upon as the cause of the renal injury. Both Sellards[23] and Peabody[24] in their studies of the chronic nephropathies reach the conclusion that the acid intoxication developing in these conditions is a retention acidosis and that the accumulation of non-volatile acids is not responsible for the kidney injury.

An analysis of the experiments which have been presented in the present study show that animals may have a severe type of chronic kidney injury that is largely localized in the glomeruli without developing an acid intoxication which can be detected by a depletion in the alkali reserve of the blood or by a reduction in the tension of alveolar air carbon dioxide. These animals show a slight retention of blood urea and a moderate reduction in the output of phenolsulfonephthalein. The minimum output of the dye in a 2 hour period for the naturally nephropathic animals has been 52 per cent. The kidneys of these animals show an epithelial element which is well preserved histologically and does not show any acute degenerative change. When these animals are anesthetized their response to the anesthetic as compared with normal animals shows the acid-base equilibrium of the naturally

[20] Porges, O., and Leimdörfer, A., Ueber die Kohlensäurespannung des Blutes in pathologischen Zuständen. III Mitteilung. Ueber die Kohlensäurespannung bei Nierenerkrankungen, *Z. klin. Med.*, 1913, lxxvii, 464.

[21] Straub, H., and Schlayer, Die Urämie eine Säurevergiftung, *Münch. med. Woch.*, 1912, lix, 569.

[22] Barcroft, J., The respiratory function of the blood, Cambridge, 1914.

[23] Sellards, A. W., The essential features of acidosis and their occurrence in chronic renal disease, *Bull. Johns Hopkins Hosp.*, 1914, xxv, 141.

[24] Peabody, F. W., Clinical studies on the respiration. II. The acidosis of chronic nephritis, *Arch. Int. Med.*, 1915, xvi, 955.

nephropathic animals to be clearly unstable, for these animals rapidly develop an acid intoxication while the control animals maintain their normal acid-base equilibrium. Furthermore, when the acid-base equilibrium of these naturally nephropathic animals is only slightly altered in the direction of an accumulation of acid ions the animals become anuric and fail to respond to a variety of diuretic substances. In the control animals which are able to maintain their normal acid-base equilibrium during the period of anesthesia these diuretic substances induce a marked increase in the formation of urine. The development of the anuria by the nephropathic animals during the period of anesthesia which coincides with the occurrence of the acid intoxication has been constantly associated with an acute degeneration of the convoluted tubule epithelium and without the development of any acute injury to the vascular tissue of the kidney.

From these experiments it would appear that in the naturally acquired kidney injury of the dog in which the chronic pathology is largely confined to the glomeruli, the injury is not due to an acid intoxication. The experiments furthermore show that when such a kidney is subjected to an agent which leads to the formation and accumulation in the blood of acid bodies, the epithelium rapidly degenerates, and that with this degeneration the functional capacity of the kidney is arrested.

CONCLUSIONS.

1. The naturally acquired chronic glomerulonephropathies of the dog are not due to an acid intoxication.

2. Such an injury renders the acid-base equilibrium of the animal unstable and susceptible to an agent such as an anesthetic which tends to induce an acid intoxication.

3. When naturally nephropathic animals are anesthetized by Gréhant's anesthetic, the principal anesthetic ingredient of which is chloroform, the animals develop an acid intoxication, and become anuric and non-responsive to diuretic substances.

4. The development of the anuria has been constantly associated with swelling, vacuolation, and necrosis of the convoluted tubule epithelium.

5. In the kidneys of these animals there occurs an accumulation of fat which is largely confined to the ascending limbs of Henle's loops and which shows a quantitative relation with the degree of acid intoxication.

EXPLANATION OF PLATES.

PLATE 49.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 5, Table II. The glomerulus, *a*, has been to a large extent converted into a mass of connective tissue. The capillaries of the glomerulus have become obliterated and adherent to the thickened capsule. Surrounding the capsule is an area, *b*, of periglomerular fibrosis. At *c* is shown the acutely swollen and vacuolated convoluted tubule epithelium which is becoming necrotic. This animal after becoming anesthetized developed an acute acid intoxication, became anuric, and failed to show any diuretic effect from theobromine or a solution of glucose.

PLATE 50.

FIG. 2. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of the naturally nephropathic animal of Experiment 11, Table II. The glomeruli at *a* show both a capsular and an intracapillary formation of connective tissue. The smaller of the two glomeruli has undergone a partial hyaline degeneration. At *b* the thickening of the capsule is marked. At *c* is shown the acutely swollen convoluted tubule epithelium which is beginning to undergo necrosis. Very early during the anesthesia this animal developed an acute acid intoxication, became anuric, and failed to show any diuretic effect from either pituitrin or theobromine.

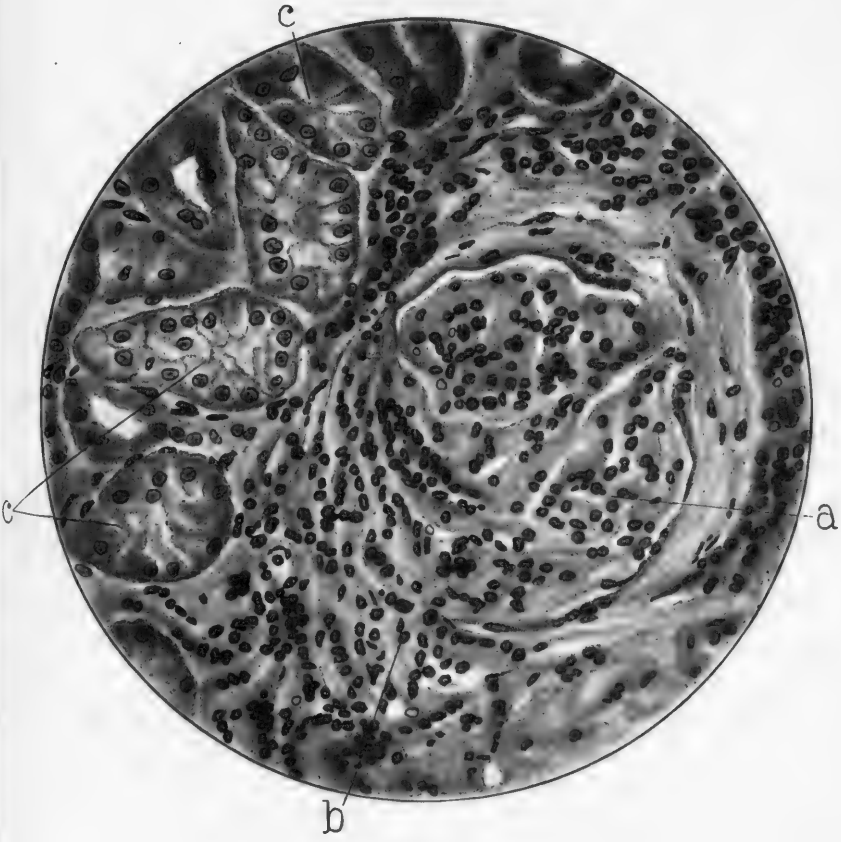

FIG. 1.

(MacNider: Nephropathic animals. I.)

FIG. 2.

(MacNider: Nephropathic animals. I.)

THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN NATURALLY NEPHROPATHIC ANIMALS AND THE EFFECT ON RENAL FUNCTION OF CHANGES IN THIS EQUILIBRIUM.

II. A Study of the Efficiency of an Alkali to Protect the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic.*

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Plates 51 to 53.

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In Part I of the present investigation, which was concerned with a study of the acid-base equilibrium of the blood in naturally nephropathic animals during the course of an anesthesia and also with the functional capacity of the kidneys of these animals, there was shown to be a relation between the depletion of the blood of its alkali reserve with the functional response of the kidney to various diuretic substances and to the development of an anuria. Naturally nephropathic animals when contrasted with normal animals as controls were shown to have an unstable acid-base equilibrium, and when the mechanism which controls this equilibrium was subjected to the action of an agent such as chloroform which tends to induce an acid intoxication, the naturally nephropathic animals very rapidly developed such an intoxication, and became anuric. The normal animals gave no evidence of an acid intoxication and in these animals the formation of urine was unaffected by the anesthetic.

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In two recent papers,[1, 2] which were concerned with the acute nephropathy induced in the dog by uranium I have been able to show not only an association between the degree of kidney injury and the severity of the acid intoxication induced by this metal, but have also shown that the intravenous use of an alkali in these animals would protect the kidney against the toxic effect of uranium and increase the efficiency of various diuretic substances. The first of these observations has been recently confirmed by the work of Goto.[3]

In the present study, Part II, an investigation will be made of the ability of an alkali to protect the naturally nephropathic kidney against Gréhant's anesthetic and to ascertain whether or not a sufficient degree of protection is obtained to enable the kidney to retain its responsiveness to diuretic solutions.

EXPERIMENTAL.

In this study a technique similar to that employed in Part I has been followed. Twenty-eight naturally nephropathic animals have been used in the investigation. Ten of these animals were used as control experiments, while the remaining eighteen animals were given an alkaline solution and furnished the basis for the deductions concerning the ability of an alkali to protect the kidney against the toxic effect of an anesthetic.

Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.

The observations on both groups of animals prior to an anesthetic are included in Table I. In this table observations are recorded on

[1] MacNider, W. deB., The inhibition of the toxicity of uranium nitrate by sodium carbonate, and the protection of the kidney acutely nephropathic from uranium from the toxic action of an anesthetic by sodium carbonate, *J. Exp. Med.*, 1916, xxiii, 171.

[2] MacNider, The efficiency of various diuretics in the acutely nephropathic kidney, protected and unprotected by sodium carbonate. II, *J. Exp. Med.*, 1917, xxvi, 19.

[3] Goto, K., A study of the acidosis, blood urea, and plasma chlorides in uranium nephritis in the dog, and of the protective action of sodium bicarbonate, *J. Exp. Med.*, 1917, xxv, 693.

two of the control animals, Experiments 1 and 2, and four of the animals which received the alkaline injection, Experiments 3 to 6. The table shows all the animals to be freely diuretic on the final day of observation prior to the use of an anesthetic. All the animals were naturally nephropathic. The urine contained albumin which varied in amount from a mere trace to 1.5 gm. per liter. Casts were present in the urine from all the animals. The urine of only one animal, Experiment 4, showed the presence of acetone bodies. The urine from this animal contained both acetone and diacetic acid. The hydrogen ion content of the blood has varied from 7.35 to 7.5. As indicated in Part I of these studies the readings do not give a true expression of the non-volatile acid content of the blood. The reserve alkali of the blood in these naturally nephropathic animals has varied between a minimum of 8 and a maximum of 8.1. The readings correlate with the determinations of alveolar air carbon dioxide which show a variation from 39 to 42 mm. These observations substantiate the conclusion made in the previous study, that the naturally nephropathic dog with kidney changes largely confined to the glomeruli does not show a depletion in the alkali reserve of the blood.

The renal function test in these animals shows a reduction in the output of phenolsulfonephthalein. The percentage elimination in a 2 hour period has varied from 54 to 64 per cent. In all the animals there is an increase in the percentage of blood urea. The animal with the highest percentage of blood urea has shown the greatest reduction in the elimination of phenolsulfonephthalein (Experiment 6, Table I). The foregoing account of observations made on naturally nephropathic animals used in this study, confirms in detail the observations made on the naturally nephropathic animals which were employed in Part I.

On the day of experiment the animals were given 300 cc. of water by stomach tube. 3 hours later under local anesthesia from a 2 per cent solution of cocaine the control animals were given intravenously 25 cc. per kilo of 0.9 per cent sodium chloride solution; while the animals which were to receive the protection against the anesthetic were given intravenously 25 cc. per kilo of a solution of sodium carbonate equimolecular with 0.9 per cent sodium chloride. The animals were then anesthetized by Gréhant's anesthetic in 60 per

TABLE I.

Observations on Naturally Nephropathic Animals Prior to Protecting the Kidney against the Anesthetic by Sodium Carbonate.

Experiment No.	Age of animal.	Weight.	Water in 24 hrs.	Urine on day of experiment.	Albumin and casts.	Acetone per 100 cc.	Diacetic acid per 100 cc.	pH	R. pH	Carbon dioxide.	Blood urea.	Phenol-sulfone-phthalein.
	yrs.	*kg.*	*cc.*	*cc.*		*mg.*	*mg.*			*mm.*	*per cent*	*per cent*
1 (control).	6	12.4	500	498	Tr. Few casts.	0	0	7.45	8.0	40	0.014	34
2 (control).	10 yrs. and 3 mos.	17.4	500	835	0.9 gm. " "	0	0	7.5	8.1	42	0.016	58
3	6	15.65	500	478	Tr. " "	0	0	7.35	8.1	40	0.015	56
4	11	17.4	500	508	" " "	11.3139	3.6456	7.4	8.0	39	0.018	64
5	13 yrs. and 2 mos.	7.85	500	611	1.5 gm. " "	0	0	7.4	8.05	40	Not made.	
6	15	18.9	500	515	Tr. " "	0	0	7.5	8.0	38	0.028	51

cent strength. 1 hour after giving the anesthetic the first observations were made on the acid-base equilibrium of the blood, the formation of urine, and the response of the kidney to various diuretic substances. The details of these observations are recorded in Table II.

A study of the control animals of Table II which received prior to the anesthetic a solution of sodium chloride shows a response on the part of these animals to the anesthetic similar to that obtained with all the naturally nephropathic animals used in Part I of these studies. The animals show a rapid depletion in the alkali reserve of the blood so that by the end of the 1st hour of the experiments the reserve alkali readings have been reduced to 7.9. At this stage of the experiments both the control animals, Experiments 1 and 2, had become anuric. The animal of Experiment 1 was given a solution of theobromine, while the second control animal, Experiment 2, was given pituitrin. The animals remained anuric and non-responsive to these diuretic substances. Half an hour after giving these solutions the alkali reserve of the blood in both the control animals was reduced to 7.85. During this period no urine had been formed. 2 hours after the commencement of the anesthesia the control animal of Experiment 1 was given 10 cc. per kilo of a 0.9 per cent solution of urea, while the second control animal was given a 1 per cent solution of theobromine. To these diuretic solutions there was no response. The blood at this stage of the experiments showed a further depletion of reserve alkali. The alkali reserve reading for the animal of Experiment 1 was 7.7, while the reading for the second control animal, Experiment 2, was 7.6. The tension of alveolar air carbon dioxide showed a proportionate reduction. The tension in Experiment 1 was reduced to 19 mm., while in Experiment 2 the tension was reduced to 15 mm. At this stage of the experiments, 2 hours after the commencement of the anesthesia, the kidneys were removed and the experiments terminated.

The histological study of the kidneys of these naturally nephropathic animals which received a solution of sodium chloride and served as control experiments shows changes similar in character to those described for the naturally nephropathic animals of Part I. The kidneys show a chronic glomerulonephropathy. The acute changes which have been induced in the kidneys by the anesthetic and which have been associated with the development of an acid intoxication

and an anuria, consist in an acute swelling and necrosis of the convoluted tubule epithelium and the deposition of large amounts of stainable fat in the ascending limbs of Henle's loops.

The following conclusions are permissible from the observations on naturally nephropathic animals which have served as control experiments: (1) A 0.9 per cent solution of sodium chloride given to a naturally nephropathic animal prior to an anesthetic has no effect in protecting the animal against an acid intoxication resulting from the anesthetic. (2) With a blood hydremic from such a solution various diuretic substances as pituitrin, theobromine, and solutions of urea are ineffective as diuretics.

Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.

A study of the animals of Table II, Experiments 3 to 6, which received solutions of sodium carbonate, shows the effect of such solutions on the acid-base equilibrium of the blood of naturally nephropathic animals and the efficiency of the solution in protecting the kidney against the toxic effect of the anesthetic. These experiments when compared with the control animals demonstrate that the use of the carbonate solution conferred sufficient protection against the anesthetic to prevent the animals from becoming anuric during the development of an anesthesia. As will be seen from a study of Table II, all the control animals at this early period of the anesthesia had become anuric.

1 hour after the commencement of the anesthetic the carbonate animals continued to remain diuretic, the flow of urine in the respective animals varying between one to two drops per minute. The alkali reserve of the blood at this period of observation as a result of the use of the carbonate solution has shown an increase in alkali above the normal. The readings vary from 8.25 to 8.3. With the alkali reserve increased to this degree the animals were given either a solution of caffeine or pituitrin to test the functional response of the kidney. Two of the animals, Experiments 3 and 5, responded to these diuretics by an increased formation of urine, while the two remaining animals, Experiments 4 and 6, though failing to respond to the

diuretics by an increase in the formation of urine, had a flow of urine of the same rate that existed before the use of a diuretic solution.

At the second period of observation, 1½ hours after the commencement of the anesthesia, the two animals of Experiments 3 and 5, which had shown a diuretic effect from pituitrin showed but a slight reduction in their alkali reserve and remained diuretic. In these animals the alkali reserve had only been reduced from 8.3 to 8.2. The two animals of Experiments 4 and 6, which had shown no diuretic effect from either caffeine or pituitrin, showed a reduction in the alkali reserve of from 8.25 to 8 and from 8.3 to 8.1. These variations in the rapidity with which the alkali reserve is used up by the anesthetized animals would indicate that the animals which continued to form urine, but which showed no increase in the output of urine from the diuretic solutions were forming during the anesthesia a larger amount of acid than were the animals which not only remained diuretic, but retained their responsiveness to diuretic substances. The tolerance for an alkali as indicated by the rapidity with which the alkali reserve is depleted is greater in the animals non-responsive to diuretics than in the animals in which the use of the diuretic solutions resulted in an increased formation of urine.

At the final period of observation, 2 hours after the commencement of the anesthesia, two of the animals which had received the carbonate protection prior to the anesthetic remained diuretic (Experiments 3 and 5). These animals had a reserve alkali reading of 8 and 7.95. The tension of alveolar air carbon dioxide for the first animal was 32 mm. and for the second animal 34 mm. The animals of Experiments 4 and 6, which also received the carbonate protection, at this stage of the experiment showed a rapid depletion in their alkali reserve and an associated decrease in the tension of alveolar air carbon dioxide. The animal of Experiment 4 had an alkali reserve of 7.85 and a tension of alveolar air carbon dioxide of 24 mm. The readings for the animal of Experiment 6 were practically the same. The alkali reserve had been reduced to 7.85, and the carbon dioxide tension to 21 mm. Both of these animals had become anuric.

At this stage of the experiments the animals were again given diuretic solutions. The animals of Experiments 3 and 5 were given respectively a solution of theobromine and a 0.9 per cent solution of

TABLE II.

Protection of the Naturally Nephropathic Kidney against the Toxic Effect of an Anesthetic by Sodium Carbonate.

Experiment No.	Na_2CO^3 or NaCl solution.	Anesthetic (Grédhunt's).	Urine per min.	pH 1 hr. after anesthetic.	R. pH 1 hr. after anesthetic.	Urine 1 hr. after anesthetic.	Diuretic.	Urine per min.	pH 1½ hrs. after anesthetic.	R. pH 1½ hrs. after anesthetic.	Urine 1½ hrs. after anesthetic.	Diuretic.	Urine per min.	pH 2 hrs. after anesthetic.	R. pH 2 hrs. after anesthetic.	Carbon dioxide 2 hrs. after anesthetic.	Urine per min.	Fat in renal epithelium.
		per cent	*gtt.*			*gtt.*		*gtt.*			*gtt.*		*gtt.*			*mm.*	*gtt.*	
1 (control).	NaCl sol. 25 cc. per kg.	60	0	7.35	7.9	0	Theobromine 1%.	0	7.35	7.85	0	Urea sol. 0.9%.	0	7.2	7.7	19	0	V.L.*
2 (control).	NaCl sol. 25 cc. per kg.	60	0	7.4	7.9	0	Pituitrin 0.5 cc.	0	7.25	7.85	0	Theobromine 1%.	0	7.2	7.6	15	0	"
3	Na_2CO_3 sol. 25 cc. per kg.	60	2	7.65	8.3	2	" 0.5 "	3	7.4	8.2	1	Theobromine 1%.	1	7.35	8.0	32	2	Tr.
4	Na_2CO_3 sol. 25 cc per kg.	60	3	7.5	8.25	2	Caffeine 1%.	2	7.3	8.0	0–1	Glucose sol. 20%.	0	7.3	7.85	24	0	L.
5	Na_2CO_3 sol. 25 cc. per kg.	60	1	7.5	8.3	1	Pituitrin 0.5 cc.	2	7.4	8.2	1	Urea sol. 0.9%.	1	7.3	7.95	34	2	Tr.
6	Na_2CO_3 sol. 25 cc. per kg.	60	1	7.65	8.3	1	" 0.5 "	1	7.4	8.1	1	" " 0.9%.	0	7.35	7.85	21	0	L.

* V. L., indicates very large amount; L., large amount.

urea. To these diuretic substances the animals were responsive. In both experiments the output of urine was increased from one to two drops per minute. The animals of Experiments 4 and 6, which had become anuric, were given either a 20 per cent solution of glucose or a 0.9 per cent solution of urea. These solutions were of no diuretic value. The animals remained anuric.

The histological examination of the kidneys of the animals which have been successfully protected against the toxic effect of the anesthetic by a solution of sodium carbonate shows the type of chronic glomerular pathology which has been previously described. The epithelium of the convoluted tubules shows only a slight degree of swelling and albuminous degeneration. The epithelium is not vacuolated, and the cells show no advanced degenerative changes indicative of a beginning necrosis. The cell cytoplasm stains uniformly and the nuclei take an intense stain (Figs. 1 and 2).

The kidneys of the animals which have shown an early protection against the anesthetic but which later in the experiments have shown a lack of protection by failing to respond to diuretic solutions and by finally becoming anuric, have like the control animals developed an acute swelling, vacuolation, and necrosis of the convoluted tubule epithelium, and have shown a large amount of fat in the ascending limbs of Henle's loops (Fig. 3).

DISCUSSION.

An analysis of the results obtained in the eighteen naturally nephropathic animals which received a solution of sodium carbonate in an attempt to protect them against the toxic effect of an anesthetic shows that based upon the efficiency of the protection the animals may be divided into two groups. All the animals for an hour after the development of a state of anesthesia remained diuretic. This is in striking contrast to the control animals that received the solutions of sodium chloride. At this period of the experiments all the latter animals had become anuric. After this first hour period, however, the animals which received the carbonate solution while remaining diuretic show a variation in the degree of protection conferred by the carbonate in that twelve of the eighteen animals which constitute one

group were responsive to diuretic substances and showed an increased formation of urine when the solutions were employed, while four of the total number of animals representing the second group gave no diuretic response to the same diuretic solutions. It is important to note that at this period of the experiments the reserve alkali of these animals had undergone a depletion, yet the depletion was not below the point of a normal hydroxyl ion content. We cannot, therefore, ascribe this lack of functional response to an acid intoxication in the sense that a sufficiently large amount of hydrogen ions had been liberated during the anesthesia to reduce the alkali reserve below the point of normality. During the remainder of the experiments these animals which early in the anesthesia failed to show an increase in the formation of urine from diuretic substances, show a rapid depletion in their alkali reserve, and become anuric.

The group of animals that received the carbonate solution and were effectively protected against the toxic effect of the anesthetic maintained for a longer period during the anesthesia the increase in the hydroxyl ion content of the blood. At the end of the experiments these animals showed a reserve alkali which was not depleted below 7.95. During the anesthesia and at the termination of the experiments the animals were not only forming urine but they were responsive to diuretic substances.

The present investigation has shown that naturally nephropathic animals may be protected in varying degrees against the toxic effect of an anesthetic by the use of an alkaline solution and that a failure to protect such a kidney during an anesthesia is associated with a rapid depletion of the blood of its alkali reserve and the development of an acid intoxication. This change in the acid-base equilibrium of the blood in these animals has in turn been associated with an acute swelling and necrosis, particularly of the convoluted tubule epithelium, and the development of an anuria. From this observation there is no evidence which would justify the conclusion that the increase in hydrogen ions acting as such upon the epithelial element of the kidney is the cause for the acute swelling and necrosis of the epithelium. The actual way in which an increase of hydrogen ions leads to an injury of the epithelium and the mode of action of an alkaline solution in deferring or preventing this injury remains a problem for future solution.

CONCLUSIONS.

1. A 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals is not effective in preventing the development of an acid intoxication and the associated kidney injury.

2. A solution of sodium carbonate equimolecular with a 0.9 per cent solution of sodium chloride when given intravenously to anesthetized naturally nephropathic animals confers a variable degree of protection to the kidney.

3. The degree of protection conferred by the alkaline solution is associated with the ability of the solution to maintain a normal acid-base equilibrium of the blood of the anesthetized animal.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 3, Table II. It shows at *a*, a glomerulus greatly increased in size from the formation of connective tissue. The capsule is but slightly thickened. The chronic glomerular pathology was mainly of the intracapillary type of change. At *b* are shown convoluted tubules the epithelium of which stains normally and shows no swelling. At *c* are shown tubules the epithelium of which is slightly swollen and stains imperfectly. The kidneys of this animal were successfully protected against the anesthetic by a solution of sodium carbonate. The kidneys were responsive to both pituitrin and theobromine.

PLATE 52.

FIG. 2. Camera lucida drawing, Zeiss oc. 3, obj. 6. The figure is from the kidney of Experiment 5, Table II. It shows at *a* the glomerulus which is increased in size from the formation of connective tissue. Many of the capillary loops have been obliterated. At *b* is shown the thickened capsule. At *c* is shown a dense mass of periglomerular connective tissue. The epithelium of the convoluted tubules at *d* shows but slight swelling. The cytoplasm and nuclei of the cells stain well. At *e* are shown two convoluted tubules in which the epithelium is increased in size without becoming vacuolated. The kidneys of this animal were successfully protected against the anesthetic by sodium carbonate. The animal remained diuretic to pituitrin and a solution of urea.

PLATE 53.

FIG. 3. Camera lucida drawing, Leitz oc. 2, obj. 6. The figure is from the kidney of Experiment 6, Table II. The figure shows at *a* the glomerulus which is greatly increased in size from the formation of connective tissue. Many of the capillary loops have become obliterated. The tuft of capillaries is adherent to the greatly thickened capsule *b*. The convoluted tubules at *c* show an acute swelling, vacuolar degeneration, and a beginning necrosis. The kidneys of this animal were imperfectly protected against the anesthetic by a solution of sodium carbonate. The alkali reserve of the blood was rapidly depleted to a reading of 7.85, and the animal became anuric and failed to show a diuretic effect from pituitrin and a solution of urea.

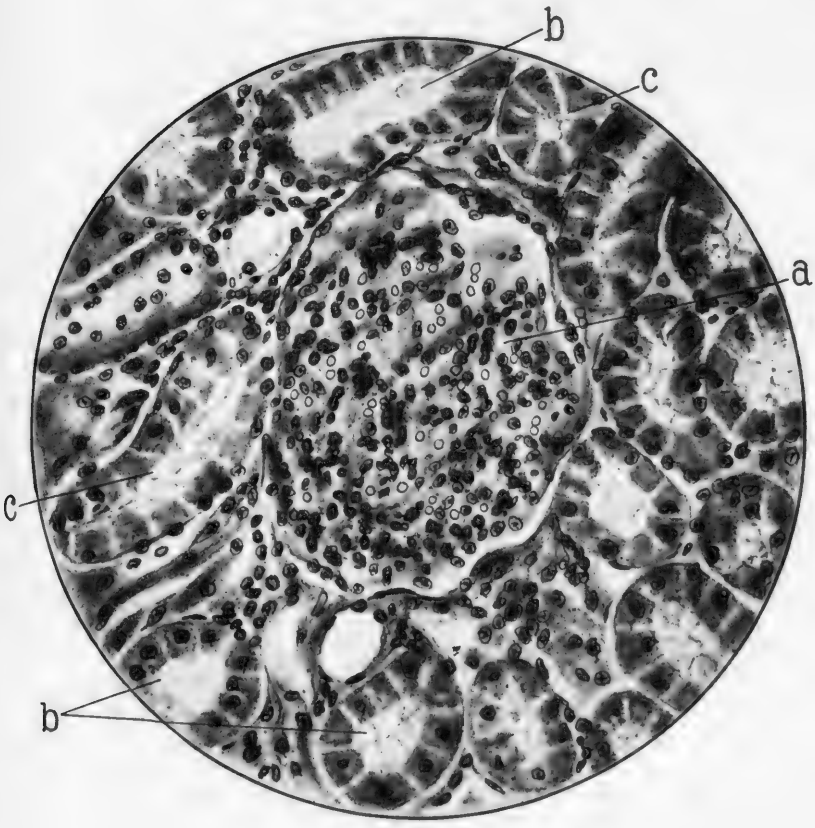

FIG. 1.

(MacNider: Nephropathic animals. II.)

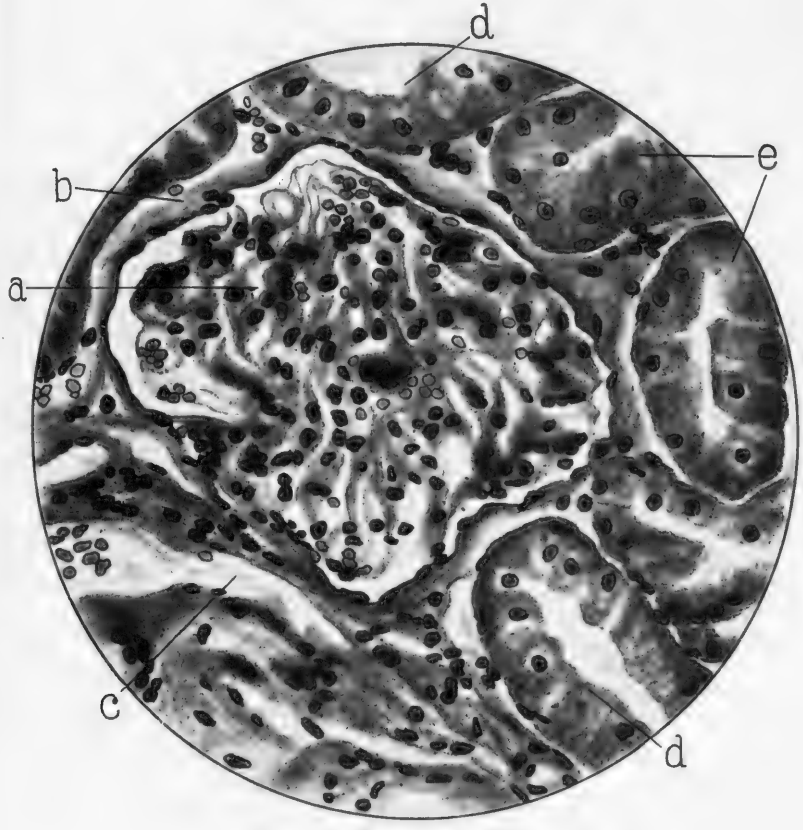

FIG. 2.

(MacNider: Nephropathic animals. II.)

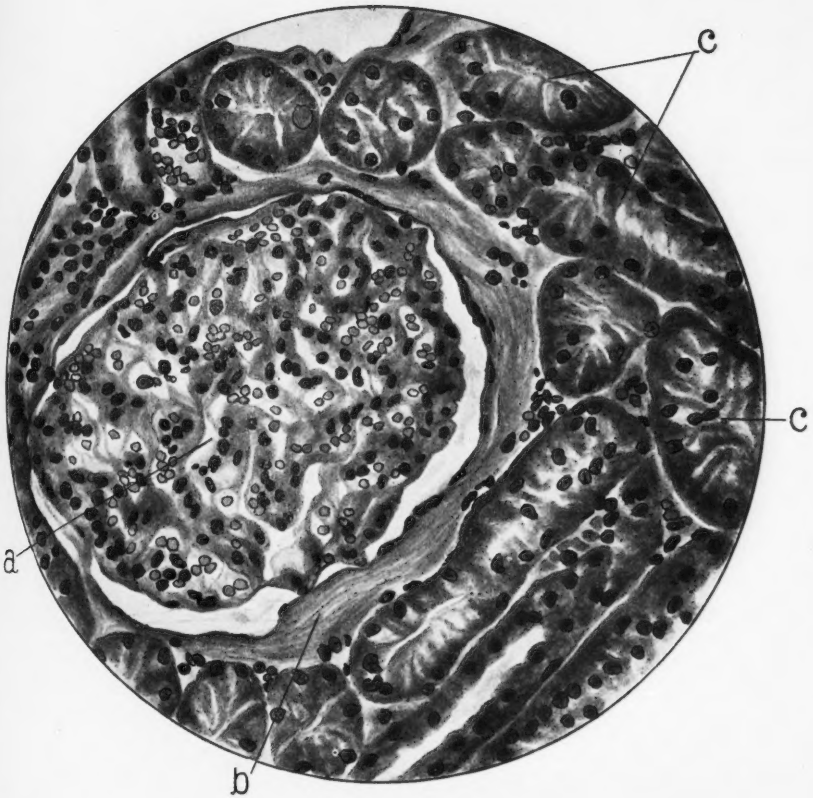

FIG. 3.

(MacNider: Nephropathic animals. II.)